

CHARACTERIZING CHRONIC LYMPHOCYTIC LEUKEMIA FOR NOVEL
BIOMARKERS OF CD40 RESPONSIVENESS USING INTRACELLULAR FLOW
CYTOMETRY

by

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Dr. Lori R. Covey

And approved by

New Brunswick, New Jersey

May, 2015

ABSTRACT OF THE THESIS

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Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia. CLL is characterized by a slow progression that generally worsens over time and currently is without a cure. A number of prognostic indicators are currently utilized to identify CLLs with a more progressive disease course. One such prognostic indicator is the responsiveness of the CLL to CD40 stimulation. CLLs that do not demonstrate a functional response to CD40L show a more aggressive clinical outcome than those that are responsive. We sought to establish a system to classify CLL cases as CD40 responsive or unresponsive using novel biomarkers by intracellular flow cytometry. This allowed us to analyze a very small number of cells from each CLL. Altogether, our work addressed optimizing in vitro culturing conditions for proliferation and viability. Also, this work analyzed a number of CD40 stimulation conditions to determine the optimal approach to analyzing CD40-mediated events in signaling. Finally, this established a

system to evaluate early intracellular signaling in the NF- κ B canonical pathway with p65 phosphorylation and I κ B α degradation as well as upregulation of CD80 following extended stimulation. Using I κ B α we were able to discern CD40 activation in CLLs that were previously characterized as unresponsive with p65 and CD80 as markers. Thus, our work extends the definition of “CD40 responsiveness” and suggests that CD40-mediated CD80 responses, which are linked to co-stimulation, may be a better predictor of CLL prognostic outcomes than early NF- κ B responses.

Acknowledgements

I would like to take this opportunity to thank Dr. Covey for her mentorship and allowing me to work in her lab. The last three years have been filled with wonderful experiences. I have been provided with a great opportunity to conduct meaningful research, attend conferences and expand my horizons. During this time I have acquired skills, memories, knowledge and appreciation for science that I will cherish for years to come.

I would also like extend my gratitude to Dr. Lisa Denzin and Dr. Ping Xie for taking the time to serve on my committee as well as their tutelage during my time at Rutgers.

To the members of the Covey Lab past and present thank you for making my time here a wonderful experience. I would especially like to thank Chris Dinh and Jim La Porta for their assistance on this project. Further thanks, Ali Saufuddin, Sean Summers, and Alex Yang for training, expertise, and making the lab a genuinely enjoyable place.

Finally, to my family for their love and support. I am extremely lucky to be part of such a loving family. I especially want to acknowledge my Father, whose courage and determination in facing Leukemia inspired me throughout this research. To my Mother, for the encouragement and motivation she has provided me. To my older siblings, Matt and Erin, as well as their spouses, Christina and Adam thank you for being such wonderful examples. Also, to my nephews Joshua and Jack, your youthful exuberance has been an appreciated reprieve from the trials and tribulations of graduate school.

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Introduction

Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia and is diagnosed in 22 to 30 percent of all global leukemia cases.¹ In the United States, CLL accounts for about one third of all leukemia cases and generally trends in older adults with the average age at diagnosis being 72 and diagnoses before 40 being extraordinarily rare.² CLL is characterized by an accumulation of neoplastic B lymphocytes in blood, bone marrow, lymph nodes, and spleen.³ Initially, CLL was considered to originate from immature B cells that demonstrate a minimal capacity to undergo self-proliferation.⁴ However, this position has changed recently and it is thought that the disease derives from antigen experienced B lymphocytes.⁵ The vast majority of CLL cells reside in early cell cycle stages such as G₀ or G₁ and supports the long-held view that the disease is the result of cell accumulation due to defects in apoptosis.⁶ More recent evidence suggests that CLL proliferation is more prominent than previously suspected. For example it has been shown that a small number of CLL cells possess Ki-67 a marker for active proliferation in addition to MCM2, which are markers for the capacity to replicate DNA.⁷ These findings support a new paradigm whereby CLL is no longer classified as strictly an accumulative disease resulting from an apoptotic defect, but rather a highly accumulative disease with some proliferative capacity and relying largely on survival signals rather than aberrant apoptosis.^{5,8,9}

Disease progression in CLL can be quite varied between individual patients. Some will live over a decade in the absence of treatment and often die as the result of unrelated factors. In contrast, other patients receive aggressive therapy to combat the disease and still succumb rather quickly.¹⁰ Multiple staging systems have been

established to aid physicians and patients in making treatment decisions given the high variability in the disease course and outcome. The Rai staging system was the first method developed to classify CLL based on specific clinical features. The Rai system divides CLL into five parts; Stage 0 to Stage IV. All stages demonstrate lymphocytosis, which is defined as an abnormally high number of lymphocytes. Stage I features enlarged lymph nodes, while in Stage II enlargement of the spleen, liver or both is typical. In addition to complications seen in previous stages, Stage III patients show anemia while patients with Stage IV CLL are characterized by a low platelet count.¹¹ An alternative staging to the Rai system was released a few years later and separates CLL into three classifications: Stage A has a high lymphocyte count with fewer than three swollen lymphoid tissues; Stage B consists of swelling in more than three lymphoid tissues; and Stage C is consistent with anemia and/or low platelet counts in addition to swelling of lymphoid tissues and a high lymphocyte count.¹²

CLL is characterized by a number of surface markers that provide significant clinical information on disease status and progression. Typical markers of the B cell lineage such as CD19 or CD20 are utilized in conjunction with CD5 and the low affinity IgE receptor, CD23, to diagnostically characterize CLL.¹³ CD5 is a member of the scavenger receptor cysteine rich superfamily and is commonly found on T lymphocytes in addition to a small subset of B lymphocytes.¹⁴ While the role of CD5 in both T and B lymphocytes is not completely understood it has been shown that it may play a role in maintaining tolerance and inducing apoptosis in B1-a cells.¹⁵⁻¹⁷ However, there are reported cases of CD5-negative CLLs, however retrospective studies demonstrated that these cases could often be reclassified as other diseases.^{18,19} CD5-negative CLL is less

frequently described in recent reports as phenotyping techniques have improved along with improved classification of B lymphocyte disorders. CD5 is also associated with Mantle Cell Lymphoma (MCL), which shares the characteristic CD19 and CD20 B lymphocyte markers. However, MCL is typically a much more aggressive form of cancer and can be distinguished based on the fact that it lacks CD23 which is expressed on CLL.²⁰

Another marker utilized to classify CLL is the status of the B cell receptor (BCR) and whether or not it has undergone somatic mutations.²¹ The BCRs of CLL cells are surprisingly less diverse than would be expected given the wide array that can typically be generated via VDJ recombination for the heavy chain and VJ recombination for the light chain.²² It is not uncommon for analysis of different CLL BCRs to yield nucleotide and amino acid sequences that are virtually identical suggesting that antigens may influence the selection of clones that give rise to CLL.²³ The mutational status of the BCR can be used as a diagnostic tool as CLLs demonstrating mutation in the IgV region will show a clinically different outcome than CLLs without mutation. Typically, CLLs showing minimal V gene somatic hyper mutations are linked to a poor prognosis and associated with a more aggressive disease course compared to those that show mutations in the BCR.^{5,10,22,24,25} Additionally, the unmutated subgroup retains BCR signaling and also expresses high levels of other poor prognostic markers ZAP70 and CD38.^{10,26-28} ZAP70 is a tyrosine kinase that is typically found in T lymphocytes that is utilized in the TCR signaling cascade.²⁹⁻³¹ CD38 is expressed on a wider array of cells but in the B lymphocyte lineage it is typically found on bone marrow precursors and is absent on

mature B lymphocytes.³² Additionally, CD38 does not track as strongly with unmutated BCR as ZAP70 and may have a more varied expression during disease progression.³³

Culturing Peripheral Mononuclear Blood Cells (PBMCs) of CLL patients is quite difficult. Despite their ability for extended survival in vivo these cells generally undergo apoptosis rather quickly in vitro.^{34–36} This occurs in spite of the fact that freshly isolated CLL cells show no apoptosis and therefore in vitro conditions fail to simulate conditions of the tumor microenvironment that support prolonged survival.³⁷ Some promise has been demonstrated when cytokines have been added to cultured CLL cells. For example multiple studies have shown that IL-4 will reduce spontaneous apoptosis and can increase expression of bcl-2 genes.^{38,39} Also, addition of IFN- α in vitro protected CLLs from apoptosis and increased bcl-2 expression.^{40,41}

Genes from the bcl-2 family are typically expressed in CLL cases although the pattern of expression and the overall effect on cell survival (with some promoting survival while others promoting death) is variable between patients.^{42,43} Other cytokines appear to promote CLL survival in vitro by either promoting cell survival or inhibiting apoptosis. Cytokines that have been reported to prevent apoptosis in B-CLL cells include IL-1, IL-6, IL-8, IL-10, IL-13, bFGF, and IFN- γ .^{34,37,44–49} B-CLL cells produce many of these cytokines with the exception of IL-4, IL-13 and IFN- α which are provided by other cells rather than autocrine signals.³⁷ Since IL-4 and IFN- α are particularly effective at reducing apoptosis it is evident that cytokine signals originating from other cells may be capable of supporting CLL both in vivo and in vitro. A critical autocrine signal capable of preventing apoptosis in CLL is IL-7. However, IL-7 mRNA is greatly reduced after only a few hours of CLL in vitro culture and exogenously added IL-7 does not prevent

apoptosis. When B-CLL cells were co-cultured with a monolayer of human umbilical cord endothelial hybrid cells it was observed that growth on this feeder layer both prevented down regulation of IL-7 and decreased apoptosis.⁵⁰ These findings suggest that CLL cells are receiving signals in vivo that support their persistence and growth. These signals that prevent apoptosis are likely lost when CLL is cultured in vitro. Based on the effectiveness of the umbilical cord endothelial hybrid monolayer to increase in vitro persistence it seems likely that these signals may be provided from direct cell-cell contact. This is supported by studies demonstrating that CLLs with diffuse bone marrow involvement correlate to advanced disease stage.^{51–53}

It has been shown that a subset of cells in CLL will differentiate in vitro into “nurselike” cells that protect CLLs from apoptosis. This effect is also observed when culturing CLLs but, not normal B cells, in direct contact with normal PBMCs.⁵⁴ Culture of B cell malignancies other than CLL have a similar in vitro fate and quickly undergo apoptosis upon culture. However, another CD5⁺ CD19⁺ malignancy MCL has shown increased in vitro viability for up to 7 months when grown on the murine stromal line MS-5 or human mesenchymal stromal cells.⁵⁵

Further investigation of replicating the tumor microenvironment revealed a bidirectional cross talk between CLL cells and the TNF-related factors, B Cell Activating Factor (BAFF), APRIL, and CD40 Ligand (CD40L).⁸ BAFF and APRIL share two receptors BCMA and TACI while a third receptor, BAFF-R is specific for BAFF. BAFF, APRIL and CD40L share some similarity in B cell biology however there are also unique roles attributed to each.⁵⁶ Of particular interest is CD40L which engages CD40 on antigen presenting cells (APCs) such as B lymphocytes, macrophage and dendritic cells.

CD40 engagement has been shown to play an important role in activating different B cell subsets. Some consequences of CD40-CD40L engagement are increased proliferation, differentiation, isotype switching and expression of costimulatory molecules CD80/CD86.⁵⁷ However, CD40L has also been used clinically as a therapy for B-CLL with some success. Patients who received gene therapy with adenovirus recombinant CD40L showed a reduced CLL cell count and increased Fas-mediated apoptosis when exposed to Fas ligand (CD95L) expressed on CD4 T Lymphocytes.⁵⁸ This induced apoptosis contrasts with the fact that CD40L provides survival signals to B cells through the NF- κ B pathway.⁵⁹ Together these findings suggest that the outcome of CD40 engagement is dependent on the environment in which it occurs. It has also been demonstrated that CD40 engagement can upregulate antigen presentation in CLL cells underscoring the use of CD40L as a way of making CLL cells more immunogenic and therefore susceptible to immune-based treatments.^{60–63} Development of immunotherapeutic treatments for CLL would target cancer cells more specifically than other therapeutic approaches. This more targeted treatment would likely reduce serious side effects associated with less directed clinical options such as chemotherapy.

In vitro investigations of CLL have determined that some samples demonstrate CD40L-specific changes.^{64,65} However, a complicating factor in the ability to utilize CD40L effectively in immune based treatments of CLL is the fact that a percentage of CLL lack a CD40 response.^{64,65} These unresponsive CLLs typically correspond to a subset of cancers with a worse clinical outcome and can be grouped with other diagnostic markers of poor clinical outcome.⁶⁵ This CD40 independent nature of certain CLLs is not the result of lacking surface CD40 as CLL cells express CD40 at levels consistent with

normal B cells.⁶⁶ CD40L stimulation in vitro parallels to an extent the CLL microenvironment since CD40L expressing CD4 T cells are often found in areas where malignant B cells occur.^{67–69} Further evidence that CD40L plays a critical role in vivo is that B-CLL cells can express CD40L and demonstrate costimulatory function typical of T cells and patients with CLL typically have elevated levels of soluble CD40L found in their serum.^{70,71} Interestingly a CD40L deficiency has been observed in CLL where the increased availability of CD40 as a result of the high number of CLL B cells results in down modulation of CD40L on T cells.⁶⁶ This down modulation can be reversed in vitro by the use of antibody that blocks CD40L-CD40 binding.⁷²

CD40 signaling typically occurs through various pathways that are often initiated intracellularly by binding of TRAF molecules to the cytoplasmic domain of CD40.⁷³ CD40 signaling is not entirely TRAF dependent; however, as some of the downstream signaling events use adapter molecules other than TRAF to initiate downstream signaling events.⁷⁴ Two of the major pathways comprising downstream CD40 signaling are TRAF signaling that leads to the canonical and non-canonical NF- κ B pathways. These two pathways are characterized by transcription factor dimers known collectively as the NF- κ B family and the canonical pathway typically proceeds faster than the non-canonical and often occurs in response to an inflammatory signal.⁷⁵ The canonical pathway typically results in phosphorylation of the p65 NF- κ B subunit and the non-canonical results in cleavage of the p100 subunit to an activated p52 subunit.⁷⁶ NF- κ B dimers are initially retained in the cytoplasm by I κ B proteins that bind the dimeric complex and prevents DNA binding. When the attached I κ B is degraded the NF- κ B becomes capable of binding DNA and activating transcription.^{75,77}

In my thesis work I sought to establish a system that allowed for enhanced culturing of CLLs and to identify potential new biomarkers for CD40 responsiveness using intracellular phosphoflow cytometry. We evaluated the culturing of CLLs with the MS-5 stromal line which had extended in vitro culture of other leukemias as well as adding B cell survival factor BAFF to CLL media.^{55,78} This work found that MS-5 cells does not extend the culture of CLL and enhances the rate of cell death whereas, BAFF had no negative effects and increases viability of CLL cultures. To identify new markers of CD40 responsiveness in CLL we performed short stimulations and stained for phosphorylated p65. These CLLs were also evaluated for upregulation of CD80 following 2 days of CD40 ligand stimulation. Further investigation with samples that did not show a p65 or CD80 response examined whether or not we can distinguish a blockade in the NF- κ B pathway. Using antibodies to upstream components in the p65 pathway we looked for differences in these events. One such marker is I κ B α a protein that binds p65 preventing it from shuttling to the nucleus. Canonical NF- κ B signaling results in the degradation of I κ B α , which allowed for assessing if the canonical NF- κ B pathway was functional in previously characterized CD40 unresponsive CLLs. This work demonstrated that CLLs previously classified as CD40 unresponsive showed I κ B α degradation and that CD40 responsiveness is likely not the result of a blockade in the canonical NF- κ B pathway. Overall, my work revealed new approaches for analyzing small numbers of CLL cells. Furthermore, it expanded on the previous understanding of the nature of “CD40-responsive” and –unresponsive” CLL samples.

Materials and Methods

Cell Culture:

Ramos 2G6 cells and PBMCs from Chronic Lymphocytic Leukemia (CLL) patients and healthy patients were cultured in RPMI Complete consisting of RPMI 1640 containing L-Glutamine supplemented with 10% Fetal Bovine Serum, 1% penicillin/streptomycin. Additionally, CLLs and PBMCs that were cultured for multiple days were supplemented with 0.1 µg/mL of recombinant soluble BAFF (Enzo Life Technologies) to reduce cell death. MS-5 stromal cells and were cultured with A10 media consisting of Alpha-MEM with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin 1% glutamine, 0.1% 2-mercaptoethanol, 1mM sodium pyruvate, 0.25% trypsin. CD40L expressing 293 HEK cells were grown in DMEM supplemented with 10% Fetal Bovine Serum, 1% penicillin/streptomycin, and 1% glutamine.

Isolation of PBMCs from Buffy Coat:

RPMI 1640 was added to Buffy Coat at an equal volume in 50mL conical tubes. Diluted buffy coat was then layered over Ficoll-Plaque Plus (GE Healthcare Biosciences) at a ratio of 3 to 2. Tubes were then spun down at 900 x g for 30 minutes with no brake. PBMCs were then counted and either placed in culture or frozen down in 500uL of freezing medium consisting of 10% DMSO in Fetal Bovine Serum.

Isolation of B Cells from PBMCs by Negative Selection:

PBMCs were thawed and counted by a hemocytometer. Cells were then spun down at 900 x g for 10 minutes and supernatant was completely aspirated. Cells were resuspended in 40uL separation wash buffer (1x PBS pH 7.2, 0.5% Bovine Serum Albumin, 20mM EDTA) per 1×10^7 cells. 30uL hapten antibody cocktail was added for

every 1×10^7 cells. Cells were incubated for 45 minutes at 4°C . 30uL of separation wash and 20uL anti-hapten magnetic microbeads were added per 10^7 cells. Cells were incubated for 90 minutes at 4°C .

An LS column was placed in a MidiMacs Separator and prepared by adding 3mL of separation wash buffer, which was allowed to elute into a FACS tube. Cells were added to the column and washed through with 3mL three times into FACS tubes. Cells were spun down, combined in RPMI complete and counted. Stains were prepared with cells set aside before and after separation to examine efficiency. Remaining cells were incubated at 37°C for 30 minutes and stimulated for desired assay.

Preparation of MS-5 Feeder Layer:

MS-5 cells were grown to near confluence in a 10 cm^2 tissue culture plate. A10 media was removed and cells were detached from the surface by adding Cellgro Cellstripper (Corning) to completely cover the plate and incubated for 10 min. Cells were washed in 1x PBS, spun down, resuspended in A10 media and put in a 5ml tissue culture flask. Cells were irradiated in an X-ray irradiator at 100V for 25 min (25Gy). MS-5 cells were returned to tissue culture plates in A10 and were incubated for at least one hour before cells were added for co-culture. Media was removed once cells were adherent and replaced with RPMI complete. CLL cells were co-cultured at a ratio 10 CLL cells to 1 MS-5.

Isolation of 293-CD40L Membranes

293-CD40L cells were grown in 15 cm^2 tissue culture plates until confluent. DMEM media was removed and replaced with fresh DMEM supplemented with

100µg/mL methyl alpha-D mannoside (α MD-mannoside) and incubated at 37°C for 1 h. Cells were removed from plates using 1x PBS with 100µg/mL α MD-mannoside and a cell scaraper. Cells were centrifuged at 1250 RPM for 7 min and washed twice with 1x PBS supplemented with 100µg/mL α MD-mannoside. Cells were subsequently resuspended and polytron-homogenized in 7.5 mL of ice-cold homogenization buffer (20 mM Tris at pH 7.5, 10 mM NaCl, and 0.1 mM MgCl₂) supplemented with 100 µg/mL α MD-mannoside, 0.1 mM PMSF, and 0.5 µg/mL DNase I.

Homogenized cells were layered in SW28 tubes onto a 25mL sucrose cushion consisting of 41% sucrose in homogenization buffer. Tubes were centrifuged at 26K rpm for 1 h at 4°C with minimal deceleration. Using a 15- gauge needle and a 10 mL syringe membranes were extracted from the middle band. Membranes were diluted in serum-free DMEM to a final volume of 26 mL and centrifuged in 70.1 Ti tubes for 45 min at 35K rpm and 15°C. 293-CD40L membrane pellets were resuspended in DMEM complete and stored at -80°C.

Stimulation using IL-4:

IL-4 (20 U/mL) (Peprotech) was used in some experiments to enhance B cell stimulatory responses.

Stimulation of B Cell Receptor Using F(ab')₂ Fragments:

6uL of 0.793mg/mL goat F(ab')₂ anti-human IgM (Invitrogen) was added to FACs tubes containing cells in 500uL of media. Stimulation occurred at 9.40 ug of anti-IgM per mL.

CD40L Stimulation:

B cells stimulated for rapid p65 activation or I κ B α degradation were incubated with CD40L stimulus for 10 to 20 min. B cells that were examined for longer response by upregulation of CD80 were incubated with CD40L stimulus for 48 h. CD40L stimulations were performed with either trimerized recombinant sCD40L (Peprotech), human recombinant MegaCD40L (Enzo Life Sciences) or 293-CD40L membranes. Stimulations were performed at concentrations of 1 μ g/mL sCD40L, 100ng/mL MegaCD40L, or 5 to 10 293-CD40L Membranes per B cell. Typically short stimulations were conducted with sCD40L; while long stimulations were conducted using MegaCD40L.

Permeabilization of Cells for Intracellular Staining:

Following stimulations, 60 μ L of 16% paraformaldehyde was added to samples for a final concentration of 1.9%. Cells were vortexed gently and allowed to incubate for 7 min at room temperature. Cells were then washed in 1 mL of FACS Wash Buffer. Cells were then spun down for 5 minutes at 1600 RPM and resuspended in 500 μ L of ice cold 100% Methanol (Optima). Cells were gently vortexed, placed at -20° C and were ready for staining after 20 minutes. Samples could also be stored at -20° C overnight and could be stained at a later date.

Carboxyfluorescein succinimidyl ester (CFSE) Proliferation Assay:

Cells were labeled using CellTrace CFSE Proliferation Kit from Life Technologies. 18 μ L of the provided DMSO was added to the 5mM Stock of CFSE to produce a 5 μ M working concentration. Cells were resuspended in 1x PBS at a concentration of 1 x 10⁶ cells/mL in a 15mL conical. 1 μ L of 5 μ M working concentration

was added for each mL of PBS. Stains were briefly vortexed and incubated in the dark at 37° for 20 min. Subsequently, 5 volumes of complete medium were added and stains incubated for an additional 5 min at room temperature. Stains were centrifuged at 1600 RPM for 5 min and pellets were resuspended in warm media. After 10 min 5×10^5 cells were collected, washed in FACS Wash Buffer and fixed in 300 μ L of FACS Fix as a Day 0 sample. Remaining cells were placed in culture and 5×10^5 cells were collected at subsequent 24 hour time periods and were washed and fixed accordingly.

Staining of Surface Antigens for Flow Cytometry

Surface staining was used to distinguish cell phenotype along with responses to extended responses to stimulation. Cells were collected from in vitro culturing conditions and put in FACS tubes containing about 2.5×10^5 cells. Cells were spun down in 1mL of FACS wash buffer at 1600 rpm and resuspended in 100 μ L of FACS wash. Cells were blocked with 1.0 μ g per sample of heat aggregated IgG and shaking for 10 min at room temperature. Cells were washed in 500 μ L of FACS wash buffer, spun down and resuspended in 100 μ L FACS wash. Antibodies were added at concentrations according to manufacturer recommendations or at concentrations that were empirically determined and samples incubated for 1 hour at room temperature with shaking. In instances where a secondary antibody was required the cells were washed in 1mL of FACS Wash Buffer, spun down and resuspended in 100 μ L of FACS Wash and stained the same way as with primary antibodies. Following final stains, cells were washed in 1mL of FACS wash and resuspended in FACS fix. Specific antibodies are listed in Table 1 below.

Staining of Intracellular Antigens for Flow Cytometry:

Cells that were permeabilized in methanol were stained intracellularly for relevant signaling responses. Cells were collected in FACS tubes at a concentration of 2.5×10^5 cells per stain. Cells were centrifuged at 1600 rpm for 5 min and then resuspended in 1mL of FACS Wash buffer. Cells were subsequently centrifuged again and resuspended in 100 μ L of FACS wash. Blocking was conducted by adding 1 μ g of heat aggregated IgG and shaking for 10 minutes at room temperature. Cells were washed in 500 μ L of FACS wash, centrifuged and resuspended in 100 μ L of FACS wash. Cells were stained with an amount of antibody corresponding to manufacturer either manufacturer recommended or empirically determined concentrations.

Flow Cytometry and Analysis:

All cells stained for flow cytometry were fixed in 300 μ L of FACS Fix Buffer consisting of 1% paraformaldehyde in 1x Phosphate Buffered Saline. Typically cells were stained with antibodies that were directly conjugated to a fluorochrome. Some stains utilized unconjugated antibodies and in those instances a second stain was conducted with a conjugated secondary antibody. All stains were with antibodies that were conjugated to the following fluorochromes: Fluorescein (FITC), R-Phycoerythrin (PE), Peridinin chlorophyll protein- Cyanine 5.5 conjugate (PCP-Cy 5.5) or Allophycocyanin (APC) Alexafluor-647. For analysis of stains utilizing multiple fluorochromes, single positive stains and double isotype control stains were prepared to establish compensation settings. Flow cytometry data was collected using a Beckton

Dickinson FACScalibur and CellQuest Pro software. Data was subsequently further analyzed using FlowJo 8.8.4 software.

Table 1: Antibodies Used in Intracellular and Surface Flow Cytometry Staining

Antibodies used in experiments are listed in the table below. Also shown is the manufacturer and the antibody clone.

| Antibody | Manufacturer | Clone |
|-------------------------------|--------------|--------------------|
| mIgG1 APC Isotype Control | BD Pharmigen | MOPC-21 |
| mIgG1 Biotin Isotype Control | eBioscience | P3.6.2.8.1 |
| mIgG1 FITC | eBioscience | P3 |
| mIgG2b PE | BD Pharmigen | 27-35 |
| mIgG2a Alexa Fluor647 | BD Pharmigen | MOPC-173 |
| mIgG2a PerCp Cy5.5 | BD Pharmigen | MOPC-173 |
| Anti-Human CD5 | eBioscience | UCHT2 |
| Anti-Human CD19 APC | eBioscience | HIB19 |
| Anti-Human CD20 PerCp Cy 5.5 | BD Phosflow | H1 (intracellular) |
| Anti-Human CD20 PerCp Cy 5.5 | eBioscience | 2H7 (cell surface) |
| Anti-Human CD23 Biotin | Ancell | BU38 |
| Anti-Human CD38 FITC | Ancell | AT-1 |
| Anti-Human CD40 Biotin | Ancell | BE-1 |
| Anti-Human CD69 Biotin | eBioscience | FN50 |
| Anti-Human CD80 Biotin | eBioscience | 2D10 |
| Anti-Cleaved PARP FITC | BD Pharmigen | F21-852 |
| Anti-pP65 (pS529) PE | BD Phosflow | K10-895.12.50 |
| Anti-pIKK γ (pS376) PE | BD Phosflow | N19-39 |
| Anti-I κ B α PE | BD Phosflow | MAD-3 |
| Anti-Human MHC II Biotin | Ancell | TDR31.1 |

Results

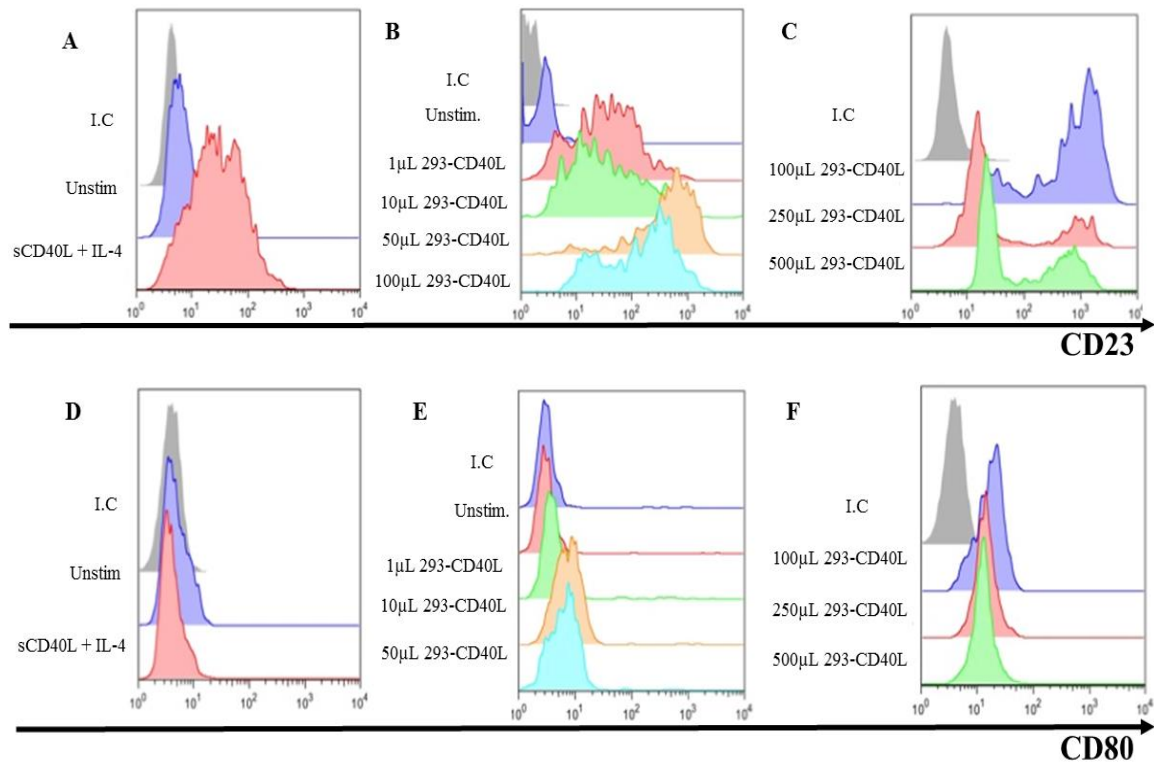
Establishing the Experimental Protocols for Measuring CD40 Responsiveness in CLL

293-CD40L Membranes Stimulate B Cells through CD40-CD40L Pathway

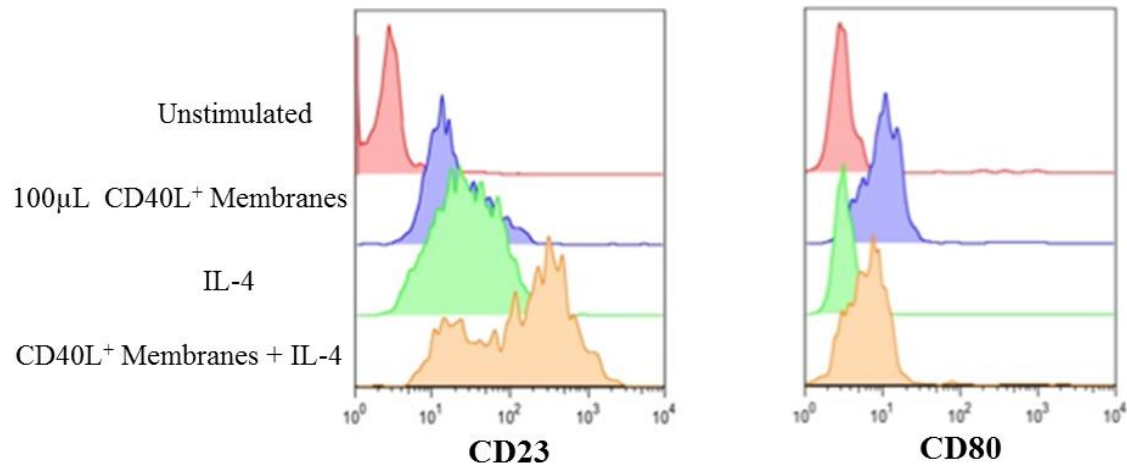
Our first goal was to determine the optimal conditions for stimulating CLL through the CD40 pathway. CD40 stimulation can be orchestrated ex vivo using various forms of its ligand and different forms of stimuli have been previously shown to induce specific downstream B cell responses.^{74,79,80} For these experiments, we tested two commercial forms of CD40 ligand; sCD40L, a trimeric recombinant soluble CD40 ligand, and MegaCD40L, which consists of two soluble CD40L trimers artificially linked via the collagen domain of adiponectin. Additionally, we utilized isolated membranes from 293 HEK cells, which were stably transfected to express CD40L (293-CD40L) on the membrane surface. To assess the effectiveness of each of these forms of CD40L for activating the CD40 pathway in general, we performed initial assays using the Ramos 2G6 immortalized human B cell line that maintains an intact CD40 signaling pathway resulting in increased CD23 and CD80 expression.^{80,81} CD23 is a low affinity IgE receptor and CD80 is a costimulatory molecule that provides activation signals to T cells through binding of CD28. We first asked whether Ramos B cells would upregulate CD23 and CD80 expression in a similar manner as sCD40L following exposure to 293-CD40L membranes for 48 h. 5×10^5 Ramos cells were stimulated with increasing numbers of 293-CD40L membranes in the presence of 20 U/ml IL-4 and the expression of CD23 and CD80 was measured by flow cytometry (**Fig. 1**). We found that CD23

expression was highest between 50 μ L and 100 μ L of 293-CD40L membranes and 20 U/mL IL-4 (**Fig. 1A-C**). In contrast, CD80 expression was much less inducible than CD23 under the same CD40L and IL-4 stimulations (**Fig. 1D**). However, based on the titration curves it was determined that ideal membrane CD40L stimulation also occurred between 50uL and 100uL (**Fig. 1E and 1F**). This corresponds to an approximate concentration of 5 to 10 293-CD40L cell equivalents per B cell. Additionally, a titration curve was also conducted for CD80 expression using identical stimulation conditions. However, CD80 was strongly upregulated in response to 293-CD40L membranes and IL-4 over 48 h. These data demonstrate that 293-CD40L membranes could effectively stimulate B cells through CD40 and that the strongest response was at 5 to 10 membranes per B cell.

Since IL-4 was used in tandem with CD40L in initial stimulation experiments we also assessed how this cytokine contributed to CD23 and CD80 responses in Ramos cells following a 48 h incubation. Cells were stimulated with 293-CD40L membranes alone, IL-4 alone or a combination of both. CD23 surface expression increased in response to both IL-4 or CD40L signals alone and the greatest increase was observed when the signals were combined (**Fig. 2, left panel**). In contrast, CD80 expression was not induced with IL-4 alone and was completely dependent on CD40 signals (**Fig. 2, right panel**)

Figure 1.**Figure 1. CD23 and CD80 expression following stimulation with 293-CD40L membranes.**

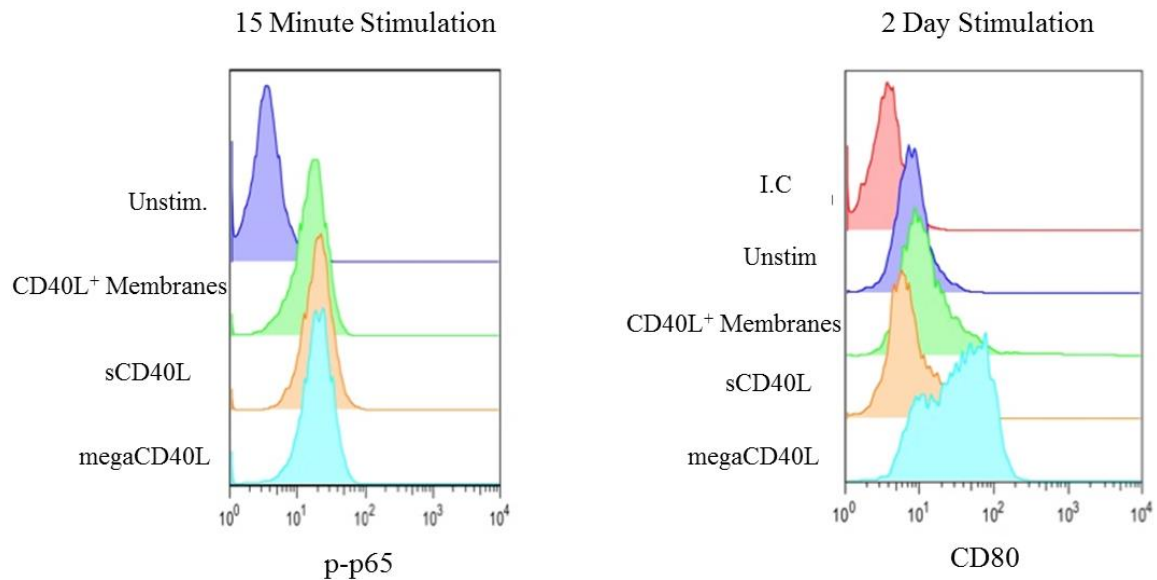
2.5×10^5 Ramos 2G6 B cells were stimulated with increasing concentrations of 293-CD40L membranes. Upregulation of CD23 (Panels A-C) or CD80 (Panels D-F) following a 48 hour stimulation with membranes. IL-4 was added at a concentration of 20U/ml to all stimulations to enhance induction of CD23 or CD80. Control stimulations were performed using sCD40L 1 μ g/mL in tandem with IL-4 and stained for CD23 (Panel A) or CD80 (Panel D).

Figure 2.**Figure 2. Effect of IL-4 on CD40L stimulation of Ramos 2G6 B cells.**

1.0×10^6 Ramos B cells were evaluated for the stimulatory effect of IL-4 on CD23 (left Panel) and CD80 response (right Panel). Ramos cells were stimulated for 48 h with 100µL of 293-CD40L membranes alone, 20U/mL of IL-4 or a combination of both stimuli.

The different forms of CD40L selectively induce B cell responses

Since we wanted to establish a system that could evaluate both short and long-term CD40 signaling responses in CLL we next needed to determine which form of CD40L stimulation was most effective in activating B cells for each time interval. For evaluating short-term CD40 responses we utilized intracellular staining with an antibody against a phosphorylated serine residue (S529) of p65, a subunit of NF- κ B that undergoes phosphorylation at multiple residues. Phosphorylation of p65 has been shown to regulate the dynamics of nuclear transport of NF- κ B and alter transcriptional activity.^{76,82} CD80 upregulation at 48 h was utilized as a measure for extended activation and was measured by flow cytometry. Stimulation of 5×10^5 Ramos B cells with 293-CD40L membranes, sCD40L, or megaCD40L was carried out for 15 min or 48 h and cells were subsequently fixed with paraformaldehyde, permeabilized with MeOH and stained with PE-conjugated antibodies. As shown in **Fig. 3** (left panel), phosphorylated p65 was equally induced in response to each stimulus. In contrast, CD80 induction was highest with MegaCD40L, while 293-CD40L membranes resulted in a greater increase in CD80 than sCD40L (**Figure 3 right**).

Figure 3.**Figure 3. Short and long term responses to various forms of CD40L stimulation.**

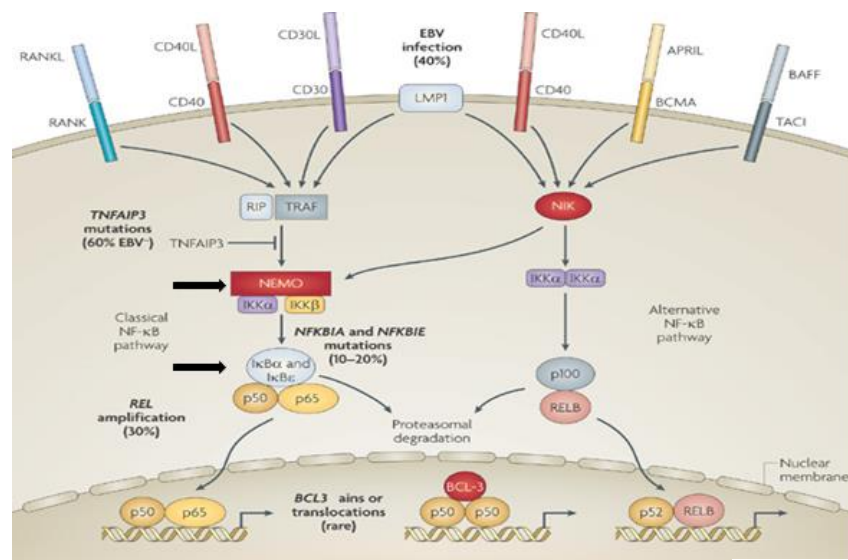
Ramos cells were evaluated for responses to different forms of CD40L stimuli. 2.5×10^5 cells were stimulated with 293-CD40L membranes (10 equivalents/B cell), sCD40L (1.0 μ g/mL) or megaCD40L (100ng/mL). Cells were evaluated for a 15 min response based on phosphorylated p65 (Ser 529) expression (left) or a 48 hour response based on CD80 expression (right).

IκBα is a second effective indicator of CD40 responsiveness

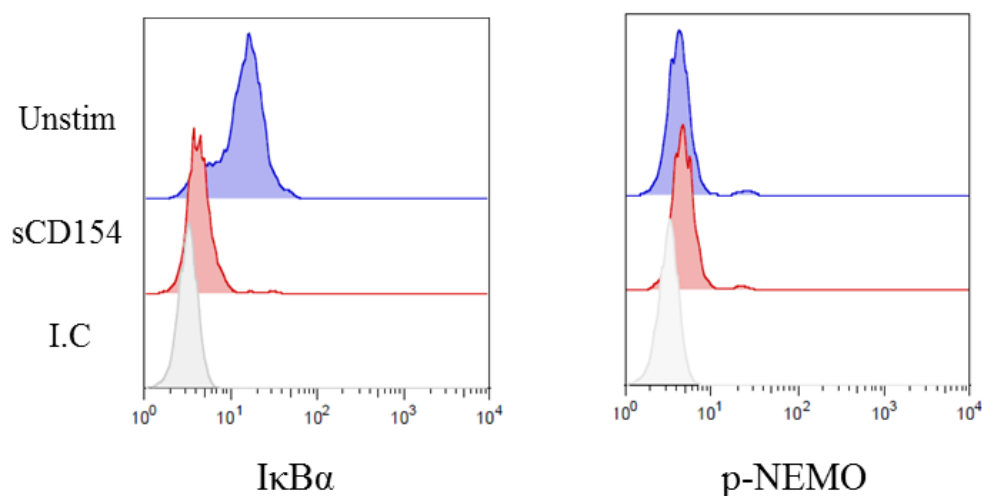
Our overall goal was to identify CD40 responsive and unresponsive CLLs and to determine if responsiveness could be mapped as an early and/or late response. However, if cells were negative for p65 activation, we wanted to determine whether signaling events upstream of p65 were also negative. We therefore examined other signaling molecules that could be targeted with antibodies and were also in the CD40 pathway. Specifically, we analyzed the responses of two molecules that are in the canonical NF-κB pathway and lie upstream of p65 phosphorylation (**Fig. 4, top**).⁸³ IκBα is bound to p65 prior to activation and in response to activation signals undergoes phosphorylation by the IκB Kinase (IKK) complex.⁸⁴ This phosphorylation is followed by ubiquitination of IκBα causing dissociation from p65 and shifts equilibrium towards nuclear localization of NF-κB.^{84–87} A second molecule is NEMO (IKK-γ) is a member of the IKK complex that phosphorylates NF-κB. The NEMO subunit does not catalyze the phosphorylation of IκBα but is an essential regulatory component of the IKK complex^{87,88}. NEMO has multiple phosphorylation sites including S376 that is phosphorylated following stimulation.

To analyze early signaling events following CD40 engagement, Ramos cells were incubated with sCD40L for 10 min, fixed, permeabilized, and incubated with anti-IκBα and anti-p-NEMO antibodies. CD40-stimulated Ramos cells expressed much less IκBα compared to unstimulated cells (**Fig. 4 bottom left**). The second marker, p-NEMO did not produce a noticeable shift in response to stimulation (**Fig. 4, bottom right**). This data indicated that IκBα degradation was measurable in Ramos cells and was a useful

marker of CD40 response. In contrast, p-NEMO was not activated and it is possible that CD40L alone is not a strong enough stimulus to produce a p-NEMO response.

Figure 4.

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**Figure 4. Molecules upstream of p65 in NF-κB canonical pathway.**

Top: Diagram depicting NF-κB signaling in B cells with arrows highlighting molecules utilized in examining CD40 response upstream of p65. **Bottom:** Ramos cells short term response was measured in response to sCD40L (1.0 μg/mL) using IκBα (Left) and p-NEMO (Ser 376) as markers (Right). Stains for IκBα and p-NEMO were compared to a type matched Isotype Control (I.C)

Optimization of CLL Culture Conditions

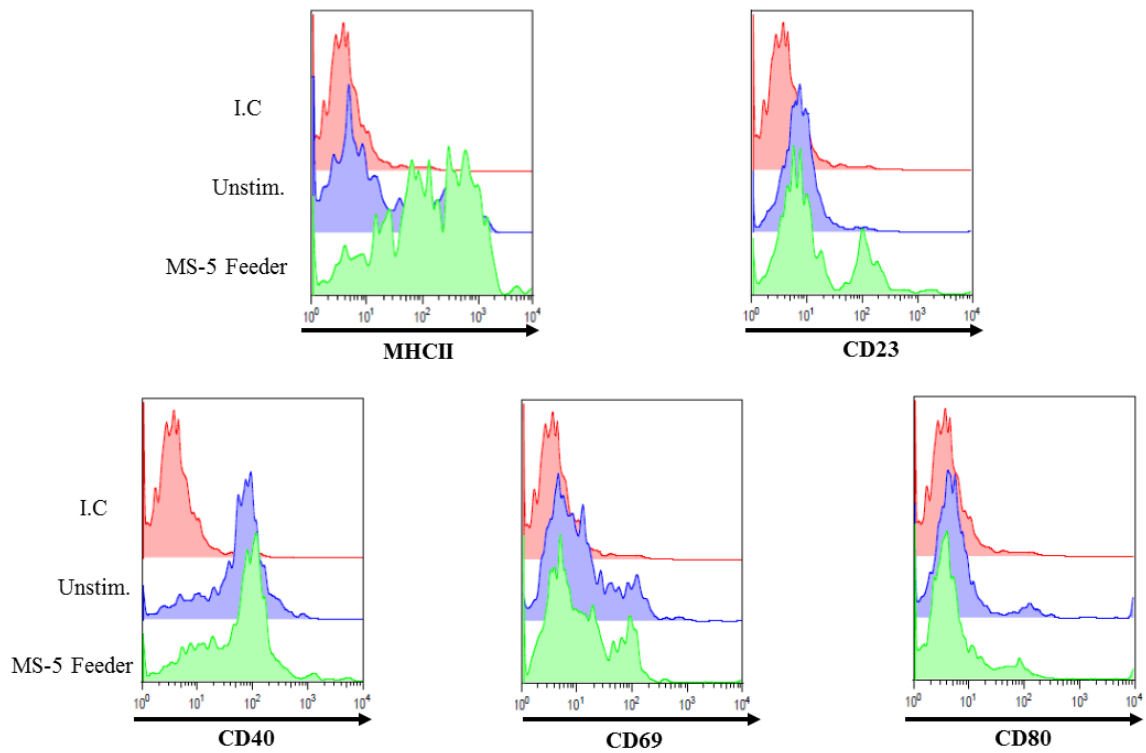
Co-culture with MS-5 cells causes CLL cell death and a non-uniform response in expression of activation markers.

CLL B cells do not survive in culture for prolonged periods and generally do not undergo substantial division.^{89,90} The murine stromal cell line MS-5 has been previously demonstrated to protect Mantle Cell Lymphoma (MCL) and Acute Myeloid Leukemia (AML) from apoptosis in culture.^{55,78} Because of these findings, we wanted to determine whether similar results would be observed by culturing CLLs with an irradiated MS-5 feeder layer. Initially, cells from Patient 3 (CLL-3) were placed in culture with irradiated MS-5 for 24 h and evaluated for changes to CD23, CD40, CD69, CD80 and MHC II molecules. A significant increase in the expression of CD23, CD69 and MHC II molecules was observed while CD80 was only slightly increased. CD40 expression itself was not changed as the result of co-culture with the feeder layer (**Fig. 5**). CD23 and CD69 are both strong indicators of B cell activation. The increase on CLL cells following co-culture demonstrated that MS-5 could activate the CLL cells. This was also supported by the slight increase in CD80 and the strong increase in MHC II both of which are used in antigen presentation by B cells.

The MS-5 feeder layer was further evaluated for effects on CLL cells after prolonged co-culturing. CLL cells were placed into culture with the feeder layer at the ratio of 10 CLL cells per irradiated MS-5 cell and evaluated at day 3 and day 10 time points for MHC II, CD40 and CD69 (**Fig. 6, top**). At day 3 an increase in CD69 and MHC II was observed. Additionally, expression of MHC II showed slightly greater expression on day 10 while CD69 remained high. CD40 expression was high initially and

showed a slight increase on day 10. Additionally, CD5 and CD20, which are markers for CLL did not change over the 10 day time period (data not shown).

We also evaluated whether the MS-5 cells could increase the proliferative capacity of the CLLs in culture by using the cell proliferation dye CFSE. Cells from P3 were stained with CFSE and placed in culture with and without the MS-5 feeder layer for 6 days. An aliquot of cells was taken at time 0 for analysis. Cells were analyzed at days 3 and 6 and a slightly increased level of proliferation was observed in cells grown on the feeder layer. However, these cultures also showed a decreased number of cells suggesting the feeder layer may increase division but also accelerate cell death (**Fig. 6, bottom**). Two additional samples were cultured on the feeder layer for three days for further examination of MS-5 induced activation. Cells from Patient 1 (CLL-1) and Patient 4 (CLL-4) were placed into culture with the feeder cells and were examined for CD23, CD69, and MHC II expression upon thawing and at Day 1 and Day 3 (**Fig. 7**). Neither sample showed an increase in CD23 or CD69 over this period. CLL-1 cells maintained expression of MHC II over the 3 day interval while CLL-4 cells displayed a decrease. The lack of a uniform response by multiple CLLs to the feeder layer in addition to the increased death of CLL cells in culture with the feeder layer led us to conclude that using the MS-5 feeder line was not an option for increasing proliferation of the CLLs.

Figure 5.**Figure 5. Activation of CLL by MS-5 Feeder Layer.**

5.0×10^6 CLLs from CLL-3 were culture with irradiated MS-5 stromal cells at a ratio of 10:1 CLL cells: MS-5 cell for 1 day and then examined for changes in expression of MHC II, CD23, CD40, CD69 and CD80. Surface protein expression was compared to a type matched Isotype Control (I.C)

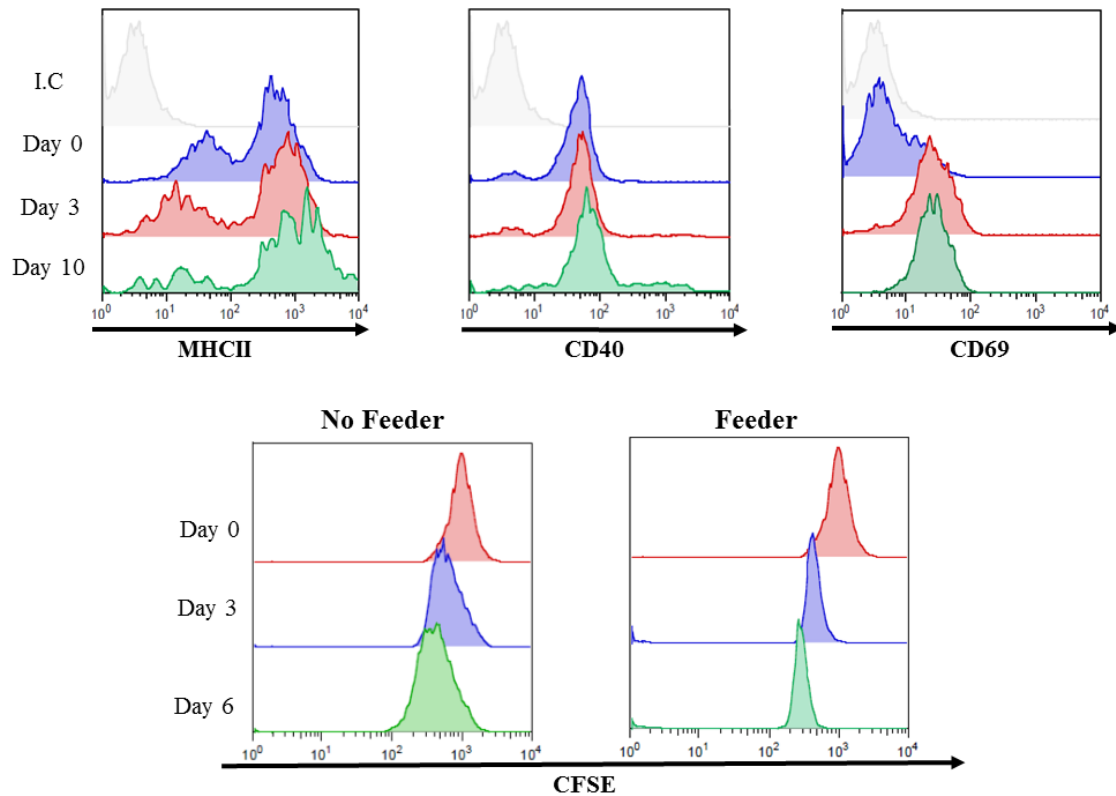
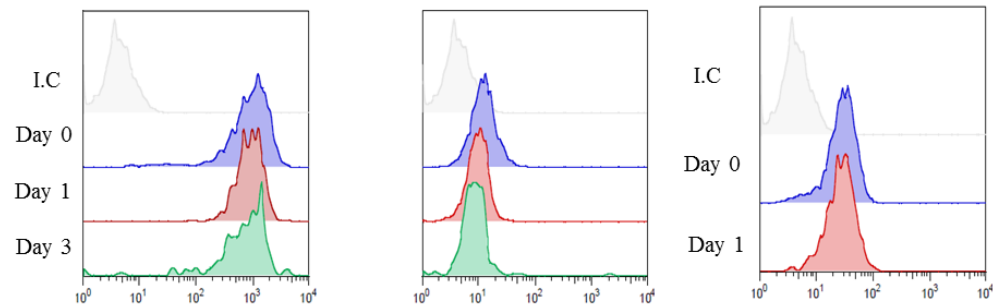
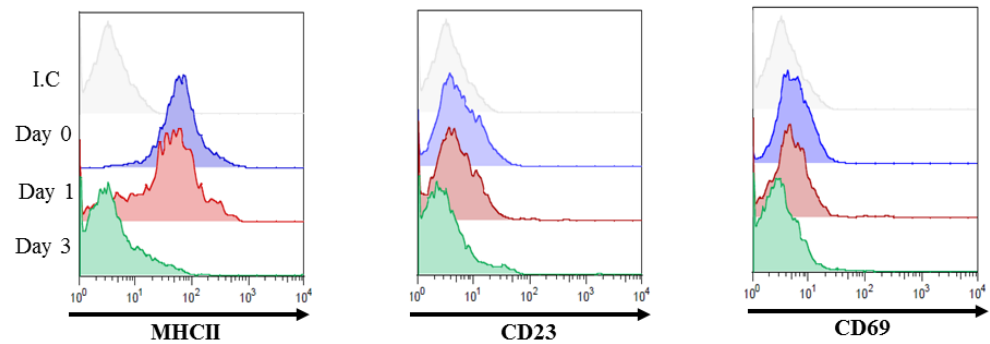
Figure 6.

Figure 6. Activation and proliferation of CLL in response to extended co-culture with MS-5 feeder layer.

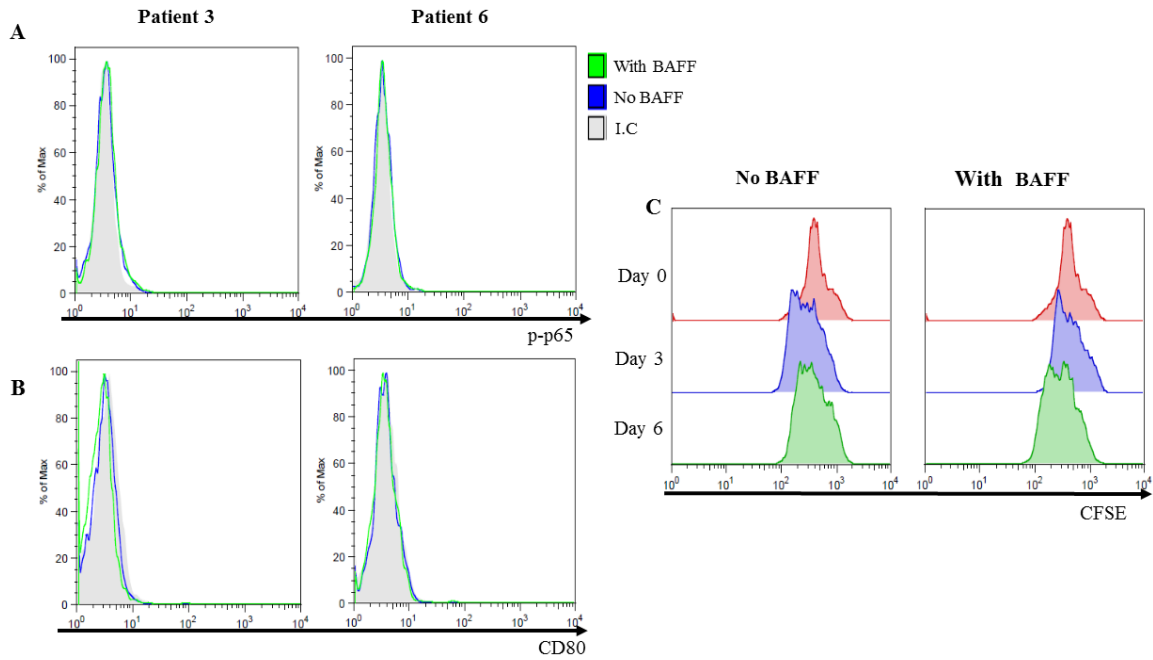
Top: 2.0×10^6 CLLs from P3 were co-cultured with MS-5 feeder cells for a prolonged period at a ratio of 10 CLLs: 1 MS-5 cell. CLLs were then stained at day 0, 3 and 10 for surface expression of MHC II, CD40 and CD69 and compared to a type matched Isotype Control (I.C). **Bottom:** 1.0×10^6 CLLs from Patient 3 were stained with CFSE proliferation dye on Day 0 and then placed into culture alone (top left) or in culture with irradiated MS-5 cells at a ratio of 10 CLLs per 1 MS-5 cell (top right). Cells were removed at day 3 and day 6 time point and subsequently examined by flow cytometry.

Figure 7.**Patient 1****Patient 4****Figure 7. CLL activation response to MS-5 feeder line is not uniform.**

CLL cells co-cultured with MS-5 Feeder cells were stained for different activation markers. CLL-1 (**top Panel**) and CLL-4 (**bottom panel**) cells were grown with MS-5 cells at a ratio of 10 CLL cells per 1 irradiated MS-5 cell. CLLs were stained following thawing and after 1 and 3 days in culture for MHC II, CD23 and CD69.

BAFF increased the viability of CLL cultures

Since MS-5 cells did not prove effective at promoting B cell survival we decided to evaluate B cell-activating factor of tumor necrosis factor (BAFF) for use in CLL cultures. BAFF has been shown to extend survival of normal B cells and CLLs in vitro and is thought to contribute to CLL survival in vivo via nurse-like cells.^{8,9,53,54,56,91,92} Also, BAFF provides survival signals to CLLs through the canonical NF- κ B pathway and has been previously shown to upregulate CD80 in normal B cells.^{9,93} We evaluated whether CLLs responded to soluble recombinant BAFF similarly to what was observed following stimulation with CD40L. 2.5×10^5 CLLs from Patients 3 and 6 (CLL-6) were stimulated with 100 ng/mL of BAFF for 15 minutes and then fixed and permeabilized and stained for anti-p-p65. Neither CLL-3 nor CLL-6 demonstrated any increase in p65 activation following stimulation with BAFF (**Figure 8A**). Additionally, 2.5×10^5 CLL-3 and CLL-6 cells were incubated with 100 ng/mL BAFF for 48 h and evaluated for CD80 upregulation. These conditions did not result in increased CD80 surface expression on CLL-3 or CLL-6 (**Figure 8B**). Additionally, we evaluated BAFF for the ability to enhance proliferation of CLL in vitro. 1.0×10^6 CLL-6 cells were stained with CFSE and then placed in culture with or without 100 ng/mL of BAFF. A fraction of the cells were taken for a day 0 sample. Subsequently, cells were removed at day 3 and day 6. CLL-6 cells cultured with BAFF did not show a significant increase in proliferation over those that were cultured without BAFF (**Figure 8C**). Since, BAFF did not alter CD80 upregulation and has been shown to enhance survival of CLLs we decided that experiments that required prolonged culturing such as 48 h stimulations, would be done in the presence of BAFF.

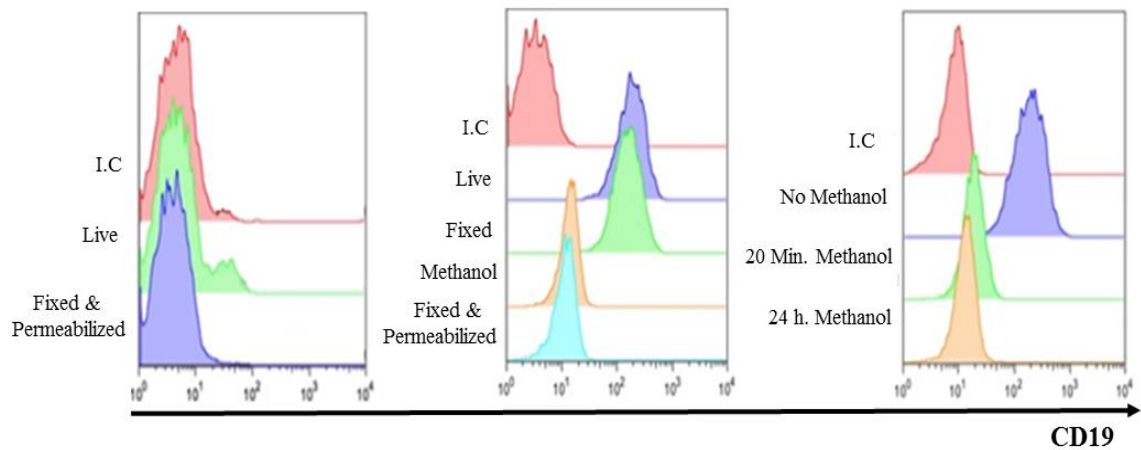
Figure 8.**Figure 8. Activation and proliferation of CLLs in response to BAFF.**

A. 2.5×10^5 CLL-3 and CLL-6 cells were incubated with BAFF (100ng/mL) for 15 min and stained for p65 activation. **B.** 2.5×10^5 CLLs were incubated with BAFF (100ng/mL) for 48 h and stained for CD80 surface expression. **C.** 1.0×10^6 CLL-6 cells were stained with CFSE and incubated with (left) or without (right) 100ng/mL BAFF.

Methanol permeabilization of cell membranes eliminates effectiveness of CD19 antibody.

Analysis of intracellular signaling pathways via flow cytometry requires intracellular antibody staining. This process requires cells to be fixed with paraformaldehyde following stimulation and subsequent membrane permeabilization with methanol to allow antibodies to bind their intracellular target. However, samples such as healthy PBMCs are a heterogeneous mixture of cell types that require the use of an additional antibody that is specific to the cell type of interest. For the CD40 signaling experiments we were interested in the signaling of B cells obtained from a healthy donor. The PBMC from CLL samples were over 90 percent positive for CD5 and CD19 or CD20 whereas healthy B cells account for approximately 6-10 percent of leukocytes in healthy adults.⁹⁴ To identify the B cells in healthy PBMC we used an APC conjugated antibody to CD19, which is a surface marker specific to B cells. However, we found that upon fixing and permeabilizing the PBMCs we would not detect a strong signal. We decided to examine whether the paraformaldehyde or MeOH treatment was causing our antibody to lose effectiveness by altering the epitope on the cell surface. To test the APC-conjugated anti-CD19 in various conditions we first stained 1×10^5 PBMCs that were untreated or fixed in paraformaldehyde and permeabilized overnight with MeOH. The untreated sample showed a population of cells that stained positive for CD19 whereas the fixed plus permeabilized sample showed no staining of B cells (**Fig. 9, left**). To further determine if fixation or permeabilization was causing the antibody to be ineffective, 5.0×10^5 cells from the D11 B cell line were left untreated, fixed with paraformaldehyde for 7 min, permeabilized with MeOH overnight, or fixed and permeabilized. The untreated sample and the fixed sample both showed effective CD19

staining. Whereas, samples that were treated with MeOH alone and or with MeOH and paraformaldehyde showed no CD19 positive staining (**Fig. 9, center**). Lastly we attempted to see if permeabilization time would alter the extent of staining by anti-CD19-APC. 5×10^5 D11 cells were again untreated or permeabilized with MeOH for 20 min or for 24 h. The untreated D11 cells again stained positive for CD19 but both the 20 min and 24 h permeabilizations reduced the APC signal to that of the isotype control (**Fig. 9 right**). Therefore, we concluded that the APC conjugated anti-CD19, that had been previously determined to be usable with permeabilized cells by the manufacturer (BD), was not able to be used as an agent with any cells that had been MeOH treated. We were able to obtain an antibody designed for intracellular staining that was specific to the cytoplasmic region of the B cell marker, CD20.

Figure 9.**Figure 9. Methanol treating cells eliminates the effectiveness of CD19 staining.**

Efficacy of APC conjugated CD19 antibody was tested following fixation and permeabilization techniques. PBMCs that were untreated and fixed and permeabilized were stained for CD19 (left). D11 cells that were untreated, fixed with paraformaldehyde, permeabilized with methanol, or fixed and permeabilized and evaluated for CD19 APC antibody binding (center), APC conjugated CD19 antibody binding of D11 cells was evaluated without methanol permeabilization or with methanol incubations of 20 min. and 24 h. (right) All stains were compared to a type matched isotype control (I.C).

Characterizing CLL Samples

CLL samples highly express B cell markers CD19 or CD20 and diagnostic marker CD5.

CLL samples were provided from the Cancer Institute of New Jersey and were obtained from peripheral blood draws of diagnosed patients. To assess the percentage of CLL cells in the individual samples, cells were stained for B cell surface markers CD19 or CD20 in tandem with CD5 a surface protein usually found on T cells although it is also found on a small subset of B cells known as B-1 cells.¹⁷ Four of the ten CLLs were stained for CD5 and CD19 together. A typical result for CD5 and CD19 staining (CLL-1) shows 93.8% of the cells were double positive for CD5 and CD19 (**Fig. 10, top**). An additional two CLLs were phenotyped using single stains to CD5 and CD19. The other four samples were analyzed with double stains for CD5 and CD20. A typical stain for CD5/CD20 (CLL-4) was 92.9% positive for CD5 and CD20 cells (**Fig. 10, bottom**). **Table 2** summarizes the results from surface phenotyping. CLLs that were stained with CD20 are indicated in parenthesis. CLL-2 and CLL-6 were stained separately for CD5 and CD19 (as shown).

Figure 10.

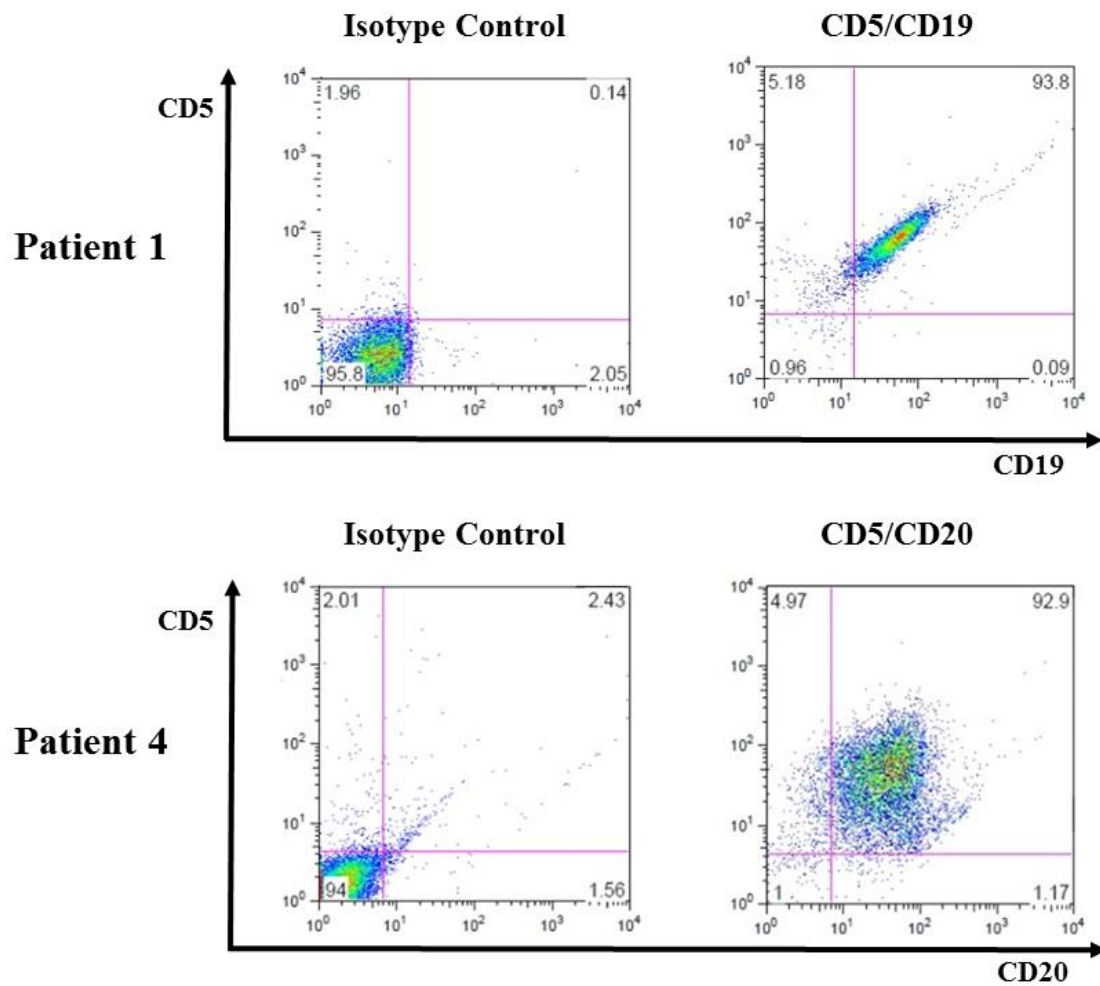
**Figure 10. Diagnostic Phenotyping of CLL**

Figure 10. Each CLL sample was stained for expression of diagnostic markers CD5 and CD19 (top) or CD5 and CD20 (bottom).

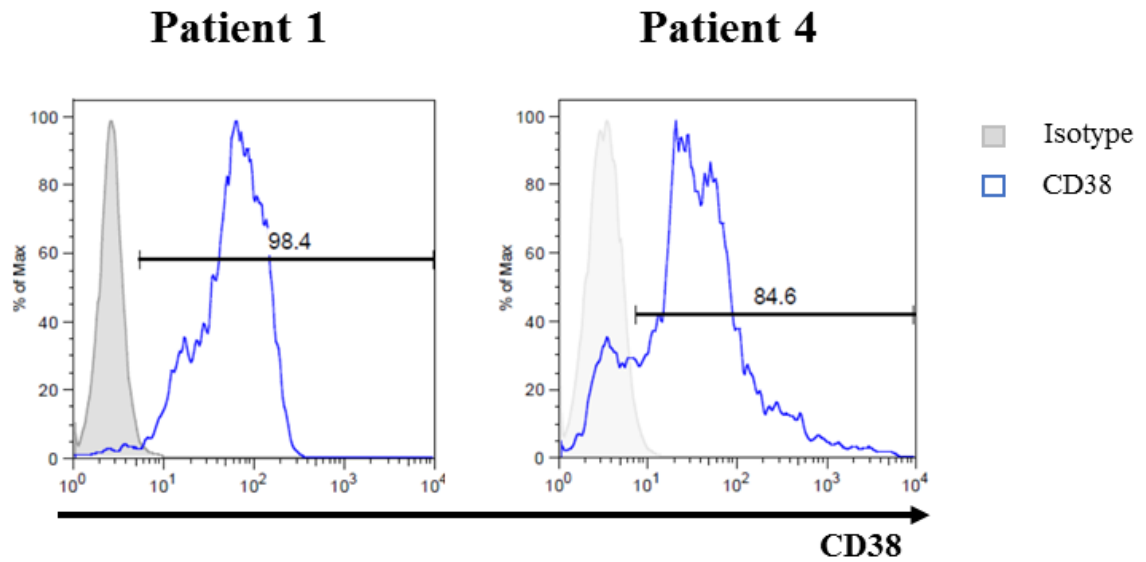
Table 2. Summary of CLL for diagnostic markers CD5 and CD19/CD20

Results for each diagnostic screen are listed below. Most samples were stained simultaneously for CD5 and CD19 or CD20 (indicated in parenthesis) and have their double positive percentage shown. Some samples were stained for CD5 and CD19 individually and have percentages for each marker listed.

| Sample | CD19/CD5 Double Positive | CD5 | CD19 |
|---------------|---------------------------------|------------|-------------|
| CLL- 1 | 93.8 | | |
| CLL -2 | | 91.7 | 92.7 |
| CLL- 3 | 92.9 (CD20) | | |
| CLL- 4 | 94.3 | | |
| CLL- 5 | 90.9 (CD20) | | |
| CLL- 6 | | 89.0 | 92.3 |
| CLL- 7 | 97.4 | | |
| CLL- 8 | 93.4 | | |
| CLL- 9 | 94.2 (CD20) | | |
| CLL- 10 | 95.2 (CD20) | | |

CLL-1 and CLL-4 are largely positive for prognostic marker CD38.

CLL-1 and CLL-4 cells were characterized for expression of the CLL prognostic marker CD38. CD38 expression on CLLs is associated with a more aggressive disease course.⁹⁵ CD38 is typically expressed across a wide array of cells but is commonly found on bone marrow precursor cells and is absent on mature B cells.⁹⁶ 2.5 x 10⁵ CLLs from CLL-1 and CLL-4 were analyzed and CLL-1 was unanimously CD38 positive, showing expression on 98.4 of cells (**Fig. 11, left**) whereas, CLL-4 showed more heterogeneous expression of CD38 while still expressing CD38 on 84.6 percent of cells (**Fig. 11, right**).

Figure 11.**Figure 11. CD38 Expression on CLL-1 and CLL-4**

2.5×10^5 CLLs were from Patient 1 (left) and Patient 4 (right) were stained with the prognostic marker CD38.

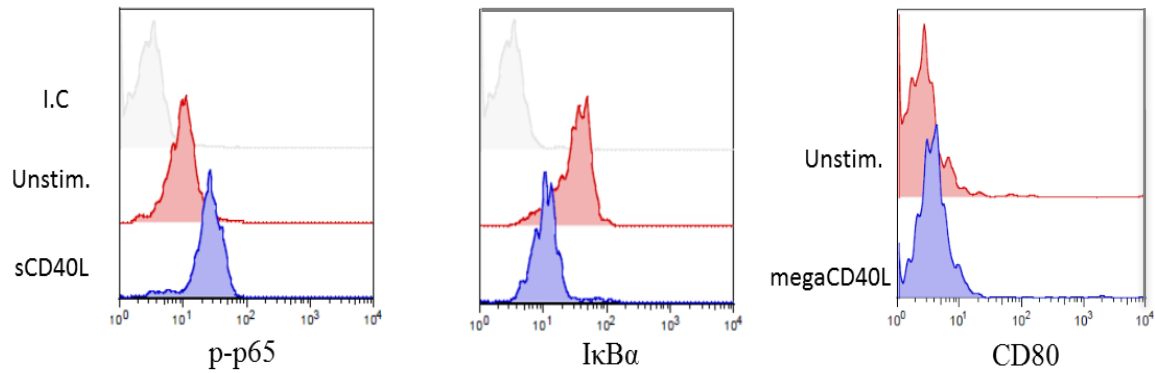
Characterization of CD40 responsiveness in CLL samples.

Prior to analyzing CLL samples, control responses were determined using PBMCs from a healthy donor. 5.0×10^5 PBMCs were stimulated with $1.0 \mu\text{g/mL}$ of sCD40L for 10 min and 15 min and stained for I κ B α and p-p65 expression, respectively. Cells were simultaneously stained with an antibody to the cytoplasmic domain of CD20 to allow for gating on B cells (**Fig. 12, left**). 5.0×10^5 PBMCs were also stimulated with 100 ng/mL of megaCD40L and stained following 2 days to observe the expression of CD80 (**Fig. 12, right**).

Experiments to analyze short-term responses of CLLs to CD40 signals were carried out by characterizing I κ B α and p-p65 and long-term responses were assessed by induced expression of CD80. Short term stimulations for p-p65 were conducted with $1.0 \mu\text{g/mL}$ of sCD40L, $9.40 \mu\text{g}$ per 500 mL of F(ab')₂ fragments IgM or a combination of both IgM and sCD40L. CLLs were fixed, permeabilized and stained for p-p65 in conjunction with an antibody to cleaved-PARP. PARP is cleaved in response to apoptosis and can also undergo differential cleavage in response to necrotic cell death as well.⁹⁷ Thus, the cleaved PARP antibody was utilized to specifically gate out cells that were apoptotic. **Figure 13** shows a comparison of the unresponsive line CLL-2 with the responsive line CLL-3. CLL-2 showed no increase in the percentage of p-p65 to sCD40L alone or in combination with IgM fragments (**Fig 13, top**). CLL-3, a strongly CD40 responsive line, had an increased from 2 percent of cells being PARP negative p-p65 positive to 18.6 percent when stimulated with sCD40L. BCR stimulation with IgM fragments alone did not induce p65 activity however, when BCR stimulation occurred in combination with sCD40L the response in CLL-3 was strongest, increasing to 28.9

percent positive for cells that were PARP negative and p65 activity (**Fig 13, bottom**). Collectively, three of the ten lines showed strong responses to sCD40L stimulation to characterize them as positive. Patient 4 and 5 additionally showed strong increases in p65 activity following CD40 stimulation. CLL-4 increased 8 percent positive for PARP negative p65 activity to 13.9 when stimulated with sCD40L while CLL-5 showed an increase of 7 percent. They did not however, show a strong response to a combined BCR and CD40 stimulations as with CLL-3 (data not shown).

Long term CD40 responsiveness was measured by stimulating 2.5×10^5 CLLs with 100ng/mL of megaCD40L for 48 h. Cells were fixed, permeabilized and stained with antibodies to cleaved-PARP and CD80. Samples from four of the patients were also stimulated through the BCR using 9.4 $\mu\text{g/mL}$ of F(ab'2) IgM fragments and with megaCD40L and F(ab'2) IgM together. CD40 responsiveness was characterized the same way as p-p65 using four quadrant graphs and MFI for PARP negative CLLs. An example of CD40 unresponsive line is shown in **Fig. 14, top panel**. Here, CLL-2 cells were characterized using both CD40L and BCR stimulation and as shown, cells did not respond strongly with either. CLL-3 was only characterized with megaCD40L and again was strongly positive as with p-p65 (**Fig. 14, bottom**). A summary of the response to each marker is shown in **Table 3**. Interestingly, cells from CLL-4, which was CD40 responsive as demonstrated by p65 activity, failed to show an increase in CD80 following 48 h of stimulation. Conversely, CLL-9 cells, which were negative for p65 activity increased expression in CD80 following CD40 stimulation.

Figure 12.**Figure 12. CD40L response in healthy primary B cells.**

CD20⁺ PBMCs were stimulated with 1.0 μ g/mL sCD40L for 15 minutes and evaluated for increased p65 expression (left) or for 10 minutes and evaluated for reduction of IkB α (center). CD19⁺ PBMCs were stimulated with 100 ng/mL megaCD40L and evaluated for increased CD80 expression.

Figure 13.

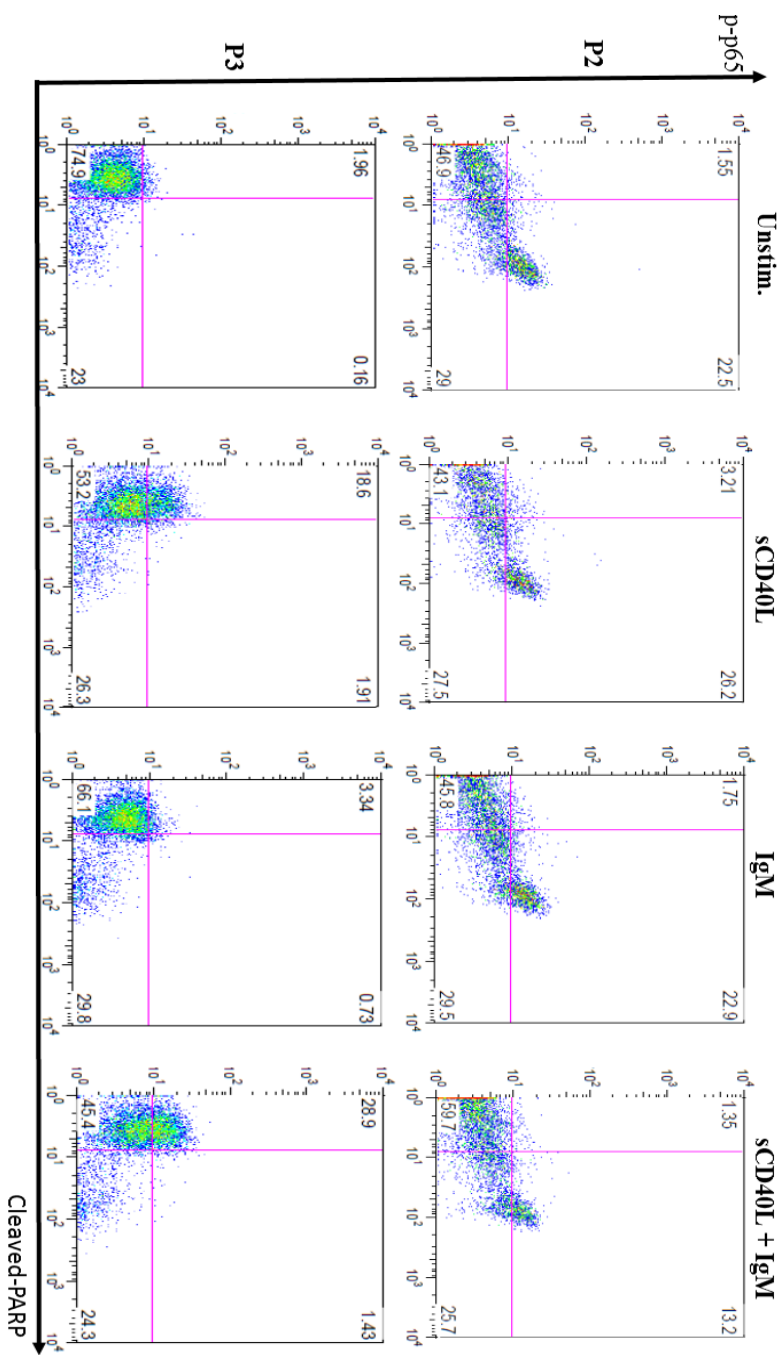


Figure 13. Short term p65 response to CD40L stimulation in responsive and unresponsive CLL

Stains were performed to distinguish between CD40 responsive and unresponsive CLLs in short term stimulations for p-p65. 2.5 x 10⁵ cells per stain were left unstimulated, or stimulated under one of the following conditions; with 1.0µg sCD40L, 9.40µg F(ab')₂ anti-human IgM per 500µL of media, or with both sCD40L and F(ab')₂ anti-human IgM. Samples were double stained with antibodies to cleaved-PARP and p-p65. Patient 2(top) shows a CD40 unresponsive line. Patient 3 (bottom) shows a CD40 responsive line.

Figure 14.

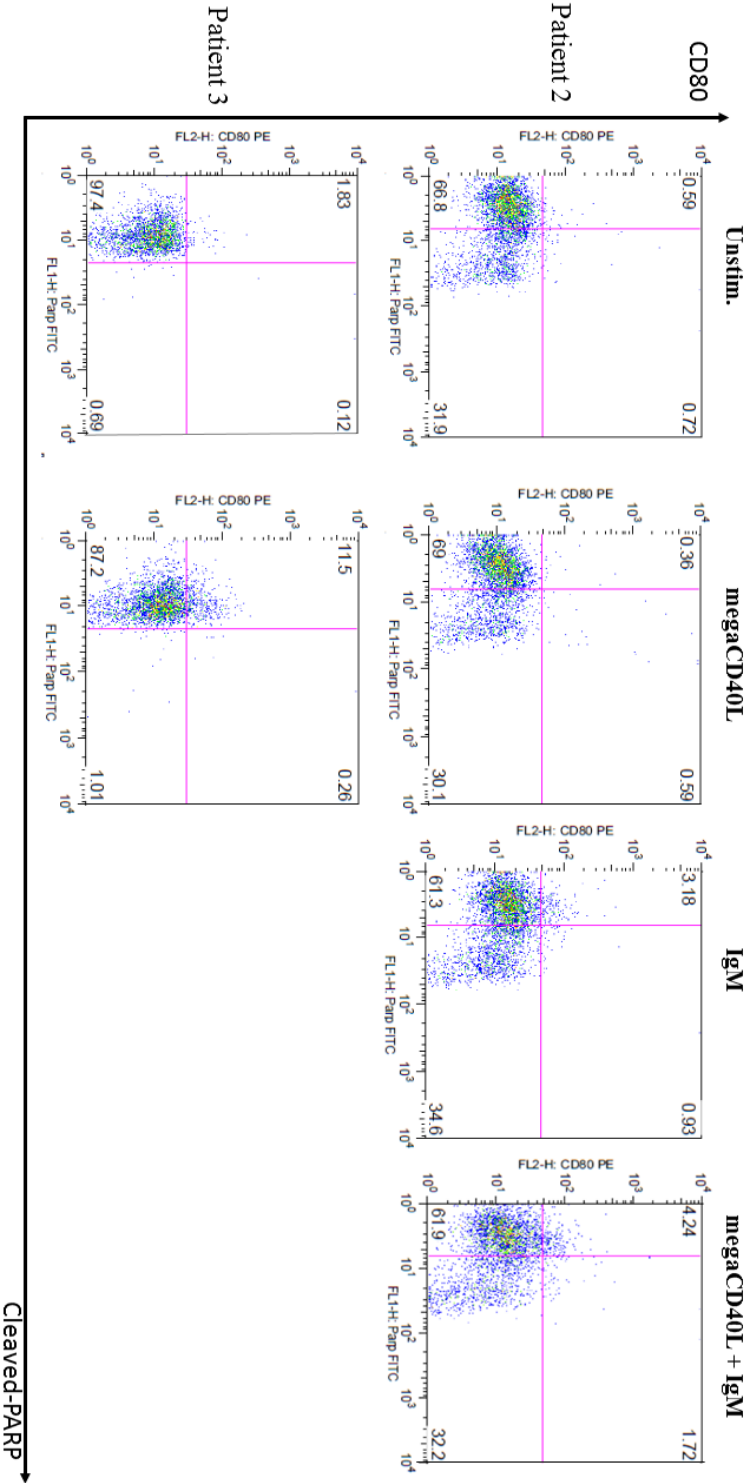


Figure 14. Long term CD80 response to CD40L in a responsive and unresponsive CLL

Stains were performed to distinguish between CD40 responsive and unresponsive CLLs in long term stimulations for upregulation of surface CD80. 2.5×10^5 cells per stain were left unstimulated, or stimulated under one of the following conditions; with 100 ng/mL megaCD40L, 9.40 μ g F(ab')₂ anti-human IgM per 500 μ L of media, or with both megaCD40L and F(ab')₂ anti-human IgM. Samples were double stained with antibodies to cleaved-PARP and CD800. Patient 2(top) was stained following stimulation with all 3 conditions and was CD40 unresponsive. Patient 3 (bottom) was only stimulated with megaCD40L and shows a CD40 responsive line.

Table 3. Summary of CLL CD40L response with each marker as well as CD38 percentage

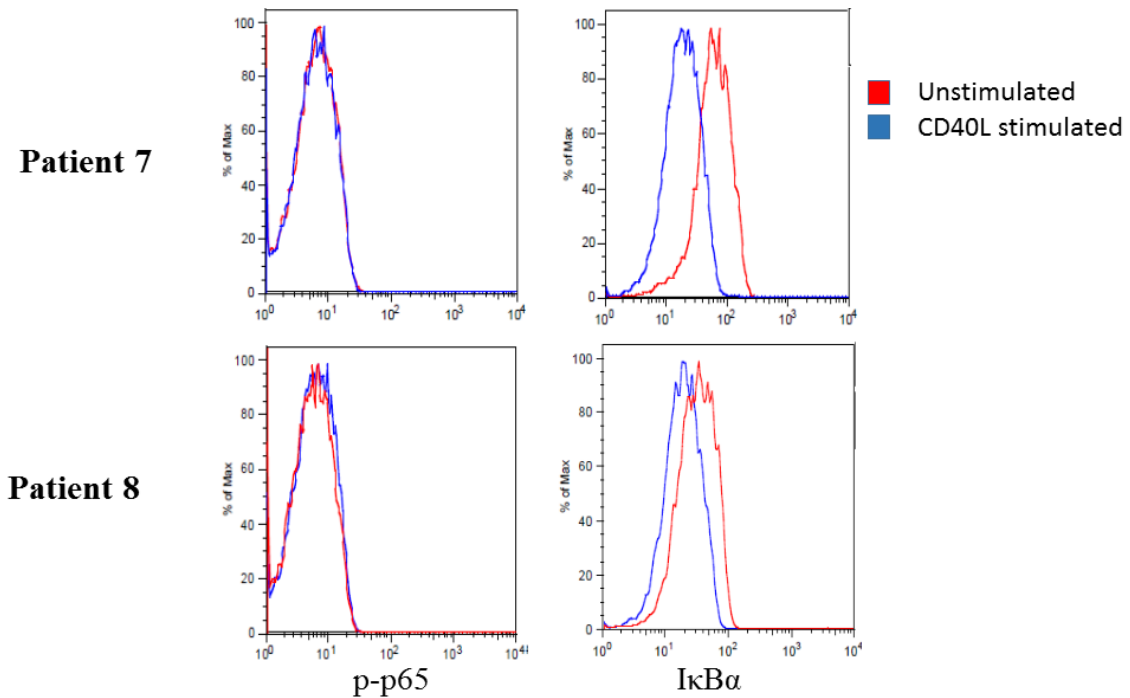
CD40 response for p65, I κ B α and CD80 in each CLL is indicated below. Also listed is CD38 percentage for CLLs stained with an anti-CD38 antibody.

| | p-p65 | I κ B α | CD80 | CD38 |
|---------|-------|-----------------------|------|------|
| CLL- 1 | - | NP | - | 98.4 |
| CLL- 2 | - | NP | - | |
| CLL- 3 | + | NP | + | |
| CLL- 4 | + | NP | - | 84.6 |
| CLL- 5 | + | NP | + | |
| CLL- 6 | - | NP | - | |
| CLL- 7 | - | + | - | |
| CLL- 8 | - | + | - | |
| CLL- 9 | - | + | + | |
| CLL- 10 | - | - | - | |

NP = Not Performed

IκBα is a better indicator of short term CD40 response than p-p65

After screening all of the CLLs for p-p65 and CD80 responsiveness we decided to examine whether we could use another signaling marker that was upstream of p65 activation. CLL-7 and CLL-8 cells were stimulated with sCD40L and stained for p-p65 and IκBα. Both CLLs had previously been characterized as negative for p-p65 when stimulated with 293-CD40L membranes (data not shown). This time we stimulated 5.0×10^5 cells from CLL-7 and CLL-8 with 1.0 μg/mL of sCD40L and stained for cleaved-PARP in tandem with p-p65 or IκBα. Both CLL-7 and CLL-8 did not show any increase in p-p65 following 15 min of stimulation however, both showed a reduction in IκBα with CLL-7 showing a substantial decrease from 71.3% positive for IκBα in PARP negative cells to 25.3% positive (**Fig. 15**). CLL-9 and CLL-10 cells were also characterized in this manner and CLL-9 cells showed a very modest reduction in IκBα while p65 did not show any change (data not shown). This data establishes that IκBα is a strong indicator of CD40 responsiveness in CLL and demonstrated a response in CLLs previously deemed CD40 unresponsive.

Figure 15.**Figure 15. IκBα is a stronger indicator of short-term CD40L responses than p65.**

2.5 x 10⁵ CLLs were stimulated for 10 min (IκBα) or 15 min (p-p65) with 1.0 μg/mL of sCD40L and stained with antibodies to cleaved-PARP and either IκBα or p-p65. Patient 7 (top) and Patient 8 (bottom) were gated on cleaved-PARP negative cells.

Discussion

The primary goal of this research was to establish a protocol to classify CLL samples for CD40 responsiveness by identifying signaling molecules that potentially have clinical significance as biomarkers using as an assay phosphoflow cytometry. This work included optimization of both CD40 stimulation and CLL culture conditions in order to maximize CLL growth and responses in vitro. The experimental focus was twofold: first to optimize a procedure for stimulating CLLs and second to evaluate early and late CD40 responses as defined by activation of the canonical NF- κ B pathway and expression of CD80.

Optimizing CLL growth and survival

MS-5 cells: Improving CLL culture conditions remains an ongoing area of research as CLLs undergo rapid apoptosis in culture despite having been shown to lack markers of apoptosis upon culturing.^{35,37,50} Previous studies indicate that the mesenchymal stromal line MS-5 can extend survival of MCL and AML malignancies in vitro.^{55,78} Our finding that MS-5 cells increased proliferation but also enhanced apoptosis in the CLL cultures was in sharp contrast to what was reported for other leukemias. This difference may correspond to disparities between the leukemias with respect to survival since CLL frequently has better clinical outcomes with longer survival times compared to MCL and AML where rapid disease progression is common.^{10,98,99} The observation that MS-5 did not activate different CLLs equally may reflect a fundamental difference in the various samples. It is possible that activation by MS-5 may

relate to CD40 responsiveness. It should be noted that a CD40 responsive CLL showed strong activation cultured with MS-5 cell while a CD40 unresponsive CLL did not undergo apoptosis when cultured with MS-5 cells. A third CLL that also was CD40 responsive albeit showing only p65 activation and not increased CD80 expression, also did not respond to MS-5 cells. CD40 responsive CLLs are thought to be more reliant on signals from the microenvironment than unresponsive CLLs.⁶⁵ This serves as a possible explanation as to why the response to MS-5 cells was different between CLLs. Assessing our data altogether it was ultimately decided that MS-5 cells were not a good option for co-culture since increased cell death was observed along with an uneven ability to activate different CLLs.

BAFF: Previous research has determined that BAFF and APRIL signals within the tumor microenvironment enhance the survival of CLLs.⁸ CD40 responsive CLLs are more proliferative in vitro and can be rescued from apoptosis with additional signals such as BAFF, APRIL and CD40L. The enhanced survival provided by BAFF, APRIL, and CD40L occurs through the canonical NF- κ B pathway by activating p65.⁹ Additionally, BAFF has been shown to upregulate CD80 expression in primary B cells after 48 h of stimulation.⁹³ Surprisingly, we did not observe activation of p65 or increased CD80 expression in CLLs cultured with BAFF as would be expected. This included results from a CLL sample that showed strong p65 and CD80 responses to CD40L ligation. It is possible that the lack of activation in the CD40 responsive CLL resulted from culturing techniques. Cells from this CLL were placed into culture and analyzed for CD40 responsiveness and frozen down. Remaining cells were then thawed and cultured with BAFF for these experiments at a later date. Subsequently, we did not observe increased

proliferation in a CLL cultured with BAFF for 6 days by CFSE staining. The CLL that was characterized for proliferation in response to BAFF was subsequently characterized as unresponsive to CD40L. In more recent experiments we have confirmed that BAFF plus megaCD40L stimulation of CLLs leads to enhanced expression of CD80 (data not shown). We are still in the process of confirming the status of p65 in CD40 responsive CLLs grown with BAFF. Since CLLs that are CD40 responsive receive multiple related proliferation and survival signals from the tumor microenvironment it is possible that CD40 unresponsive CLLs do not require any of these signals and fail to respond to additional interactions from BAFF and other molecules.^{8,9,92}

CD40 Stimulation: Our characterization of CD40 responsiveness was based on intracellular phosphoflow cytometry in addition to upregulation of CD80. In order to optimize responsiveness it was necessary to test different CD40 stimuli which have been previously examined in multiple in vitro systems.¹⁰⁰ Early models of CD40 responsiveness used monoclonal anti-CD40 antibodies to mimic CD40 ligation and demonstrated that the ability to increase proliferation, activate signaling pathways, and induce IgE secretion was likely related to the specific epitope of the antibody.¹⁰⁰⁻¹⁰⁴ More recently researchers have used soluble recombinant forms of CD40L or cells that express either anti-CD40 antibodies or membrane bound CD40L.^{100,105} The advantage of using CD40L as opposed to an antibody is that it more accurately reflects in vivo CD40-CD40L interactions.¹⁰⁰ Membrane-bound and recombinant soluble forms of soluble CD40L each provide advantages and disadvantages. Membrane-bound systems are generally cheaper than recombinant forms and more closely mimic the in vivo fluid membrane dynamics of CD40-CD40L interactions.^{100,106,107} Additional research

demonstrated that membranes from 293 HEK cells transfected to express CD40L led to the upregulation of CD80 in CLL B cells.⁷⁹ However, our use of 293-CD40L membranes as a stimulus required a substantial number of membranes that complicated flow analysis by accounting for increased background events compared to viable cells. Of particular interest is a newer system that is very similar to the CD40L-expressing 293 membranes however the cells express both a soluble form and a surface-bound form of CD40L. In experiments that compared the expansion of B cells, co-culturing with 293-CD40L-sCD40L showed 10 times greater expansion than 293-CD40L following 25 days in culture.¹⁰⁸ Our findings revealed that in response to short stimulations, the activation of p65 in Ramos B cells was similar with 293-CD40L membranes compared to the two forms of commercially available CD40L; sCD40L a trimeric recombinant form, and megaCD40L which is comprised of two CD40L trimers linked through the collagen domain of adiponectin.¹⁰⁹ We found that megaCD40L produced a stronger induction of CD80 compared to sCD40L or 293-CD40L membranes, which was likely due to increased stability of megaCD40L.

Measuring CD40 responses in CLL cells

We found that the majority of CLLs were classified “CD40-unresponsive” by failing to exhibit either a short p65 activation or CD80 upregulation in response to CD40 stimulation. A number of CLLs demonstrated only a p65 or a CD80 response. Specifically, the three CLL samples that showed only early responses and no increase in CD80 expression reflect CLLs that are functionally unresponsive to CD40. It would be interesting to observe whether this difference in short term and long term response was

the result of downstream signaling blockages. We found previously that CD80 induction was weaker than CD23 in response to sCD40L and 293-CD40L membranes. Additionally the ability to express CD80 and more recently CD95 following CD40L stimulation varies from sample to sample and is independent of p65 expression. Previous research using multiple assays for assessing CD40 responses in CLLs classified a number of samples as CD40 responsive that did not demonstrate an upregulation of CD80 or CD95 however these CLLs did express chemokines and anti-apoptotic proteins following stimulation with CD40L.⁶⁵

NEMO and IκBα: Our decision to evaluate non-responders for additional CD40-mediated upstream events led us to assess the phosphorylation of NEMO and the degradation of IκBα. Activation of both of these molecules occurs upstream of p65 phosphorylation where NEMO is a regulatory subunit of the IKK complex that targets IκBα for degradation^{87,88} and the degradation of IκBα drives the dissociation and activation NF-κB.^{76,88,110,111} Surprisingly, we observed no NEMO activation in Ramos B cells stimulated with CD40L. One possible explanation for this finding was that we needed to inhibit protein phosphatases in the cells in order to observe NEMO phosphorylation. It has been reported that PBMCs stimulated with TNF-α failed to induce NEMO activation however, when TNF was added in conjunction with the protein phosphatase inhibitor Calyculin A there was a strong response.^{112,113} TNF and CD40L are both members of the TNF superfamily and induce many similar signaling events in B cells.^{114,115} Thus, activation of NEMO in B cells in response to CD40L engagement may be highly similar to that of TNFR and require the addition of Calyculin A to produce a detectable change. Future experiments to analyze phospho-NEMO will include the

addition of Calculylin A to CD40 stimulations to potentially slow or prevent competing phosphatases from masking the activation of NEMO.

In contrast to what was observed with NEMO, we found that I κ B α expression was visibly decreased in response to CD40L molecules and revealed that a subset of previously characterized CD40 unresponsive CLLs showed a CD40 response through degradation of I κ B α . We looked at four CLLs that showed negative p65 or CD80 responses. Two of these CLLs did not demonstrate either a positive p65 or CD80 response, while the other two demonstrated activation of only one of these two markers. Three of the CLLs that showed a negative p65 or CD80 were positive for I κ B α degradation when stimulated with CD40L. Since one CLL was responsive for I κ B α and CD80 induction but not p65 activation, it seems unlikely that there signaling blockage is occurring but rather that the assay for p65 activation is less robust than that for I κ B α . These results indicate that I κ B α is possibly a better indicator of CD40 responsiveness than p65 and should be used to evaluate CLL responsiveness to short-term stimulations in future experiments.

CD38 expression: CD38 is a multipurpose type II transmembrane glycoprotein ectoenzyme that has been reported to be a poor prognostic marker in CLL.^{33,65,95} CD38 has been shown to correlate with the prognostic marker ZAP-70 but not immunoglobulin variable region mutations.^{33,65,116} Interestingly, previous research is conflicting with regard to the relationship between CD38 expression and CD40 responsiveness.⁶⁵ We examined CD38 since it is another prognostic marker that reflects a poor outcome in CLL.^{33,95} The two CLLs that were analyzed showed high CD38 expression and were both negative for CD80 induction while one demonstrated some p65 activity. These

results align with previous work that showed that CD38⁺ cells can be classified as CD40 responsive and unresponsive.⁶⁵ This suggests that the usefulness of CD38 expression as a prognostic is more nuanced and is context specific. Also, it is unclear if p65 activity alone is sufficient to designate a CLL CD40 responsive.

Future Directions

Future research will continue to seek ways to improve ex vivo culturing techniques of CLL to more closely replicate the tumor microenvironment. The current model for understanding CLL biology is that CLL is characterized by a balance of cells circulating in blood where they generally accumulate with cells found in the lymph node tumor microenvironment where they undergo proliferation or apoptosis.^{95,117,118} The tumor microenvironment provides CLLs with proliferation and anti-apoptosis signals that mediate the formation of proliferation centers.¹¹⁸ Proliferation centers are composed of CLLs, T lymphocytes and stromal cells, where CD40-CD40L and BCR signaling occur as well as other signals such as BAFF and APRIL.^{8,64,119,120} Mimicking in vivo proliferation centers through the use of stromal cell lines and stimulatory signals should enhance survival and proliferation. This has been tested using other endothelial cells such as splenic microvascular endothelial cells (SMVEC), lymphatic microvascular endothelial cells (LMVEC) and microvascular endothelial cells (MVEC), which activate CLLs and provide survival and proliferation signals.⁸ However, advances may still remain limited as most CLL samples are obtained from peripheral blood where they were already removed from proliferation centers prior to collection and may not be able to transition back. Experiments with CLLs obtained from biopsied lymph nodes and bone

marrow of patients will potentially produce samples with higher proliferative capacities. Notably this introduces additional concerns as bone marrow and lymph node biopsies are much more invasive and time consuming than collecting peripheral blood from patients. Another concern is that the BAFF, APRIL and CD40 signals provided by stromal cells are all TNF-related factors and occur simultaneously within the tumor microenvironment.⁸ This suggests that while CD40 responsive CLLs will benefit from co-culture, CD40 unresponsive lines likely operate more independent of signals in the tumor microenvironment and thus will remain more difficult to culture *ex vivo*.

Further research will attempt to discern an underlying mechanism of CD40 unresponsiveness. CD40 has been shown to induce multiple signaling cascades not limited strictly to the canonical NF- κ B pathway. These include the non-canonical NF- κ B, mitogen activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), and the phospholipase C γ (PLC γ) pathways.¹²¹ CD40 responses typically rely on immediate recruitment of TRAF molecules to the cytoplasmic domain of CD40.^{73,121} Expanded cell numbers would allow us to extend our investigation to the earliest CD40-induced signaling events in CLLs using western blotting. This may be achieved by growing cells in the presence of CD40L and IL-21, which has been shown to increase *in vitro* proliferation of CLLs.¹²²

Conclusions

An important contribution that this work makes to the field of CLL is that it has developed protocols to analyze small numbers of CLL cells for CD40 responsiveness using rapid and relatively non-invasive clinical approaches. Intracellular flow

cytometry for classifying CLLs based on CD40 response is highly valuable since it requires a relatively small number of cells and can provide a detailed look at intracellular pathways rapidly.

Finally, our findings have altered the notion of “CD40 responsiveness” by analyzing both early and late responses. We found that many of the CLLs examined did not produce consistent CD40 responses when assessed for I κ B α degradation, p65 activation and CD80 upregulation. However, we demonstrated that I κ B α degradation is a strong indicator of early “CD40 responsiveness” in CLLs. In terms of anti-tumor immunity, it is highly desirable that CLLs express co-stimulatory molecules so that they can engage and activate tumor-associated T cells. Therefore, “functionally” non-responsive CLLs, which do not effectively present tumor antigens, will likely evade approaches to increase CLL immunogenicity.

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